# STUDY ON MORPHOLOGY, PHYSIOLOGY AND FATTY ACID PROFILES OF ASCHERSONIA FOR IDENTIFICATION OF CLOSELY RELATED FUNGAL TAXA

MS. PLEARNPYT LUTHISUNGNEON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY PROGRAM

SCHOOL OF BIORESOURCES AND TECHNOLOGY

KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI

1998

ISBN 974-624-528-6

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of *Aschersonia* for identification of closely related fungal taxa

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ISBN 974-624-523-6

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|---|---|
| Thesis Title | Study on morphology, physiology and fatty acid profiles of *Aschersonia* for identification of closely related fungal taxa |
| Thesis Credits | 12 |
| Candidate | Miss Plearnpit Lutthisungneon |
| Supervisors | Asst. Prof. Dr. Yuwapin Lertwerawat |
| | Dr. Nigel Hywel-Jones |
| Degree of Study | Master of Science |
| Department | Biotechnology |
| Academic Year | 1998 |

## Abstract

Five isolated strains of three morphologically similar species of insect pathogenic fungi, *Aschersonia* from natural forest in Thailand were studied. These were *Aschersonia tubulata* -5877 , *Aschersonia tubulata* -5996 , *Aschersonia hypocreoidea* -3162 , *Aschersonia hypocreoidea* -5269 , *Aschersonia placentata* -5164. Growth was on five different solid media Malt Extract Agar (MEA) Minimal Salt Medium Agar (MSM) , M102 , Potato Dextrose Agar (PDA) and Sabouraud Dextrose agar (SDA) at 22°C. The results showed that all five strains grew better on PDA than other media. PDA was then selected for further studies of temperature response. The temperature ranges of 15, 20, 22, 25, 28 and 30 °C were used. The optimal range for growth were found between 22-28 °C with the optimal temperature at 25 °C. It was found that the sporulation depends on type of media, temperature and also strains of *Aschersonia*. The characteristics of conidia shape in all *Aschersonia*

strains studied are fusiform. Condition such as media, temperature in this study affect to size of conidia (width and length). All, five strains of *Aschersonia* express the same composition of fatty acids i.e.palmitic acid, stearic acid, oleic acid and linoleic acid. The results showed that stearic acid was produced at lowest level. In contrast linoleic acid was found accumulated at the highest level.

Keywords : *Aschersonia*/ Fatty acid/ Insect pathogenic fungi

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| หัวข้อวิทยานิพนธ์ | การศึกษาลักษณะทางสัณฐานวิทยา, ลักษณะทางกายภาพ และองค์ประกอบของกรดไขมันเพื่อใช้ในการจัดกลุ่ม *Aschersonia* |
| หน่วยกิตของวิทยานิพนธ์ | 12 หน่วย |
| โดย | นางสาวเพลินพิศ ลัทธิสูงเนิน |
| อาจารย์ที่ปรึกษา | ผศ.ดร. ยุวพิน เลิศวีระวัฒน์ |
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| สาขาวิชา | เทคโนโลยีชีวภาพ |
| ปีการศึกษา | 2541 |

## บทคัดย่อ

ศึกษาลักษณะทางสัณฐานวิทยาของราก่อโรคในแมลงชนิด *Aschersonia* ซึ่งแยกได้จากป่าไม้เมืองไทยจำนวนห้าสายพันธุ์และจัดเป็นสามกลุ่มใหญ่ดังนี้คือ *Aschersonia tubulata*-5877, *Aschersonia tubulata*-5996, *Aschersonia hypocreoidea*-3162, *Aschersonia hypocreoidea*-5269, และ *Aschersonia placenta*-5164 นำมาเลี้ยงในอาหารแข็งห้าชนิดได้แก่ Malt Extract Agar (MEA), Minimal Salt Medium Agar (MSM) , M102 , Potato Dextrose Agar (PDA) และ Sabouraud Dextrose Agar (SDA) ที่อุณหภูมิ 22 องศาเซลเซียส พบว่า *Aschersonia* ทั้งห้าสายพันธุ์สามารถเจริญได้ดีบนอาหาร PDA ดังนั้นจึงคัดเลือกอาหาร PDA ศึกษาถึงลักษณะการเจริญที่อุณหภูมิแตกต่างกันโดยเลือกใช้อุณหภูมิ 15, 20, 22, 25, 28 และ30 องศาเซลเซียส จากการศึกษาพบว่าช่วงอุณหภูมิที่ *Aschersonia* ทั้งห้าสายพันธุ์สามารถเจริญได้ดีอยู่ในช่วง 22-28 องศาเซลเซียส โดยที่ 25

องศาเซลเซียส เป็นอุณหภูมิที่ *Aschersonia* ทั้งห้าสายพันธุ์สามารถเจริญได้ดีที่สุด พบว่าลักษณะการสร้างสปอร์ของเชื้อราขึ้นอยู่กับชนิดของอาหารและอุณหภูมิที่เหมาะสม ซึ่งมีความแตกต่างกันในแต่ละสายพันธุ์ และสปอร์มีรูปลักษณะเป็นกระสวยในทุกสภาวะของอาหารและอุณหภูมิที่ใช้ในการทดลองในขณะที่ขนาดของสปอร์ (กว้างและยาว) จะแตกต่างกันเมื่ออาหารและอุณหภูมิเปลี่ยนแปลงไป เมื่อศึกษาถึงชนิดของกรดไขมันพบว่า *Aschersonia* ทั้งห้าสายพันธุ์มีองค์ประกอบของกรดไขมันที่เหมือนกันคือ กรดปาล์มมิติก, กรดสเตียริก, กรดโอเลอิก และ กรดลิโนเลอิก โดยพบองค์ประกอบของกรดสเตียริกในปริมาณน้อยที่สุดเมื่อเทียบกับปริมาณกรดไขมันทั้งหมด ในทางตรงข้ามพบว่ามีกรดลิโนเลอิกในปริมาณมากที่สุดเมื่อเทียบกับปริมาณกรดไขมันทั้งหมดเช่นกัน

คำสำคัญ (Keywords) : *Aschersonia*/ กรดไขมัน/ ราก่อโรคในแมลง

Acknowledgements

I am very grateful to Dr.Nigel Hywel-Jones, my supervisor at National Center for Genetic Engineering and Biotechnology (BIOTEC) with his constant help, encouragement and friendship. I am also grateful for my supervisor, Asst.Prof. Dr. Yuwapin Lertwerawat at KMUTT for her help and suggestion in this work and I would like to express my sincere gratitude and thanks to Assoc. Prof. Dr. Morakot Tanticharoen for her advice, support and introduction to work this experiment at BIOTEC laboratory (NSTDA). Thanks also to Somsak, Aire and Pla at BIOTEC for their kindness, help, friendship and encouragement in the laboratory. I also acknowledge the considerable financial support provided by Biodiversity Research and Training Programme (BRT) to make this study successful.

Finally, I would like to thank my parents for their understanding, encouragement and support for my study.

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# List of abbreviations

| | |
|---|---|
| °C | degree celcius |
| g | gram |
| h | hour |
| km | kilometre |
| $km^2$ | square kilometre |
| m | metre |
| µm | micrometre |
| µl | microlitre |
| mg | milligram |
| ml | milliltre |
| mm | millimetre |
| TFA | total fatty acids |

# Chapter 1

## Introduction

### 1.1 Background

Due to the great diversity of living organisms, it is necessary to arrange groups based on their shared characteristics, which traditionally have been morphological. Taxonomy is defined as the science of biological classification of living organisms including, also, an understanding of how species develop [ 1 ]. Prescott, Harley & Klein (1990) have stated that "many taxonomists have maintained that the most natural classification is the one with the greatest information content or predictive value." They further conclude that a good classification should bring order to biological diversity and even explain the function of morphological structures for a given species. Many characteristics are used in classifying and identifying microorganisms. Morphology is easy to study and analyse, particularly in eucaryotic microorganisms and the more complex procaryotes where there is a good range of characteristics to use.

Furthermore, morphological comparisons are valuable because:

- structural appearance depend on the expression of one or more genes,
- are usually genetically stable, and

- normally (at least in eucaryotes) do not vary much with environmental conditions.

Thus, morphological similarity in species is a good indication of phylogenetic relatedness. Many different morphological features can be used in the classification and identification of microorganisms. Physiological and metabolic characteristics are also increasingly useful because they are related to the nature and activity of the organisms enzymes and transport proteins. Since proteins are gene products, analysis of these characteristics provides an indirect comparison of an organisms genome [ 2 ].

The first study of fungi was begun when van Leeuwenhoek invented the microscope in the seventeenth century. Anatomical characters of fungi can be observed with a compound microscope in squash mounts, hand cut sections or microtome sections of embedded material. Gross morphological features such as fungal thallus, shape, colour and size of spore-producing structures are easily seen at low magnifications. In the late nineteenth century the life cycle and morphological characteristics were used for classification [ 3 ]. Gross morphological features were traditionally used to classify higher fungi such as the Ascomycota and Basidiomycota.

Development of advanced techniques and instruments (especially since the early 1960's) has increased the features that can be studied. Electron microscopy is important in the study of new characters and is a valuable tool for understanding evolution of organisms especially organelles such as flagellae, nuclei and nuclear

function, asci, cell wall structure, conidium ontogeny and the internal structure of mitochondria [ 4 , 5 ]. Scanning electron microscopy is also important having the advantage that only small specimens are prepared and high-resolution observation of external or exposed inner surface structure is possible [ 6 ]. This technique is very popular to resolve minute spore ornamentation and especially details of conidium ontogeny.

Increased knowledge of staining protocols allows for the histochemical localisation of certain compounds and biochemical events in structures of interest. Currently, developments with fluorescent probes and dyes allow some localisation procedures previously done with electron microscopy to be visualised with fluorescence or confocal microscopes [ 7 ].

Biochemistry is also an increasingly important technique for the modern taxonomy of filamentous fungi. Frisvad and Filtenborg (1989) [ 8 ], Frisvad and Filtenborg (1990) [ 9 ] and Frisvad (1992) [ 10 ] used chemical techniques such as Thin Layer Chromatography (TLC) and protein electrophoresis to separate species of *Penicillium* (Mitosporic state of Ascomycota: Eurotiales) while Cruickshank and Pitt (1987) [ 11 ] identified species of *Penicillium* subgenus *Penicillium* using enzyme electrophoresis. In their study they used polyacrylamide gel electrophoresis to examine the extracellular enzymes polygalacturonase, pectinolytic enzyme, amylase and ribonuclease. In contrast, ubiquinone systems have also been studied. For example Sugiyama *et al.* (1988) [ 12 ] studied the ubiquinone systems of rusts (Basidiomycota: Uredinales) and smuts (Basidiomycota: Ustilaginales) while

Kuraishi *et al.* (1991) [ 13 ] studied ubiquinone systems in *Penicillium* and related genera. Most recently nucleic acid sequencing has been used in studying the taxonomy of fungi [ 14 , 15 ] and is assuming greater importance in determining phylogenetic relationships. Fatty acid patterns may be also useful for characterization of the relationship between species. Although fatty acid analysis is useful for studying taxonomy there are limitations [ 16 ]. Different conditions induce changes in fatty acid patterns and one necessity, therefore, for studying fatty acids for chemotaxonomy is to clearly define experimental conditions [ 17 ].

Many fungi contain greater amounts (and varieties) of polyunsaturated fatty acids than do higher plants and animals [ 18 ]. In the past, fatty acids were considered for the identification of organisms. Analysis of cellular fatty acid composition is used routinely to differentiate, characterize and identify genera, species and strains of bacteria [ 19 ]. Very little taxonomic importance was, however, attached to such profiles for filamentous fungi, possibly because there were many morphological features to make use of. However, currently, work has shown that cellular fatty acid composition can be used to differentiate and identify genera, species and strains of yeasts and yeast-like organisms [ 20 ].

The fatty acid composition of a fungus can provide valuable physiological and taxonomic information as to its relationship with other fungi that may not be available from morphological characteristics. Isoenzyme analysis and analysis of fatty acids are useful for overcoming limitations with morphological characteristics. Gas chromatography (GC) can be used to characterize fatty acid profiles of lipids in

biological materials [ 21 ]. A necessary procedure associated with this analysis is lipid derivatization. This process changes the volatility of lipid components and improves peaks thus providing better separation [ 22 ].

## 1.2 Background problems to this research

The genus *Aschersonia* [ 23 ] is one insect pathogen that has been used for biocontrol [ 24 ]. It was not only studied for biocontrol but also was studied with respect to the production of pigments [ 25 ] and the distribution of chymoelastases and trypsin-like enzymes [ 26 ]. However the background information about *Aschersonia* is problematic because they have been poorly studied and much of the literature is in excess of eighty years old [ 27 ]. The modern reviewer can often only guess at what the terminology really meant as language and ideas have altered. Currently, the understanding of the taxonomy of these fungi has changed considerably. In the past taxonomic studies were often on herbarium material only and, when examined, this material was invariably dried and preserved with no record of its treatment. Subsequently, a great deal of original descriptions are based on dead material and as such these are seen, now, to bear little relationship to the living material [ 28 , Hywel-Jones, pers. comm. ]. Because of these limitations this thesis investigated the morphology, physiology and fatty acid composition to provide more information on *Aschersonia* so that our understanding of the genus can be brought up to date.

## 1.3 Outline of the research

Since most of the taxonomic literature on *Aschersonia* is over 70 years old this study was done to determine if more modern techniques using pure cultures could be used to assist in the taxonomy of the genus. Five strains representing three closely related species of the genus *Aschersonia* were used to study their morphology and physiology. Each strain was grown in five solid and five liquid media to look for the temperature and media for optimal growth of the *Aschersonia* strains Gas chromatography was used to characterize the fatty acid profiles of the five *Aschersonia* strains. The results from these studies were compared in order to separate closely related fungal taxa.

## 1.4 Benefits

The investigation of morphology, physiology and fatty acid profiles will increase the available characteristics for separating *Aschersonia* strains and related species. This will be the base for further development such as the search for new antibiotics, novel metabolites, genetic studies and the fermentation of *Aschersonia* for biocontrol screening.

# Chapter 2

## Theory: The organism

### 2.1 Microbial Taxonomy Concept

1. To make sense of the diversity of organisms, it is essential to group similar organisms and establish these groups in a non-overlapping hierarchical order.

2. Organisms can be placed in one of five kingdoms based on cell type, level of organization and type of nutrition.

3. The basic taxonomic unit is the species, which is defined in terms of either sexual reproduction or general morphological similarity.

4. Classifications are increasingly based on an analysis of possible evolutionary relationships (phylogenetic or phyletic classification) whereas they were historically based on overall similarity of morphology (phenetic classification).

5. Morphological, physiological, metabolic, genetic and molecular characteristics are all useful in taxonomy because they reflect organization and activity of the genome. Nucleic acid structure is probably the best indicator of relatedness because nucleic acids are either the genetic material itself or the product of gene transcription.

This study is interested in the *Aschersonia* anamorph state of the insect pathogenic genus *Hypocrella* Sacc. (Clavicipitales).

Domain Eukaryota
Kingdom Eumycota
Phylum Dikaryomycota
Sub phylum Ascomycotina
form-class Deuteromycetes
form-genus *Aschersonia*

Domain Eukaryota
Kingdom Eumycota
Phylum Ascomycota
Sub phylum Ascomycotina
order Clavicipitales
genus *Hypocrella*

Saccardo (1882-1925) used the nature of conidiophores and conidiomata to classify deuteromycetes (mitosporic fungi). Recently, classification systems have changed and the specific terminology of fungi is now related to the fungus life cycle. Distinct characteristics of morphology have also altered terminology and these modifications have been adopted for this thesis. The fundamental classification of the Saccardoan System is a character-based arrangement which grouped fungi according to shared or similar morphological features without any phylogenetic implication. The biology of the majority of ascomycetes species is poorly known. The ascomycete flora is also

poorly known for large portions of the world and this is especially the case in the tropics (a region noted for its high species diversity).

The universal holomorph describes the phenomenon of characteristics of pleomorphy with characteristics of morphology. The pleomorph is shown by the holomorph or the parts of all fungal life cycles that reflects the meiotic state (teleomorph) and mitotic state (anamorph) [ 29 ]. Over the years many different schemes have developed different terminology.

## 2.2 Mitosporic Fungi

The term mitosporic fungi [ 30 ] is not familiar to many people whereas Deuteromycotina, Deuteromycetes, Fungi Imperfecti, asexual fungi and conidial fungi are freely used by many mycologists. However, mitosporic fungi is a more accurate term that avoids the problems of recognizing Deuteromycotina as a distinct taxonomic grouping. While Deuteromycotina were historically considered at a taxonomic level they are now recognised to be one part of the life-cycle. Currently, the classification of fungi separates two systems based on meiosis (sexual) and mitosis (asexual). The system adapts the use of "true" generic names for sexual fungi only, while "form-genus" status is kept for the asexual state of fungi. Sexual fungi were classified by a "botanical" system while asexual fungi were classified by an "anatomical" system [ 31 ].

The meiosis part of the life-cycle involves a mother nucleus dividing into daughter nuclei in which the ploidy is lower than that of the parent nucleus and in which

recombination of chromosomes can occur. In contrast, the mitosis part of the life-cycle involves nuclear events where chromosomes are replicated and distributed equally between the daughter nuclei with no recombination of genetic material.

Mitosporic fungi have a relationship with the teleomorph in ascomycetes and basidiomycetes and have been termed anamorphs or anamorphic states of these groups. Most anamorphs of ascomycetes and basidiomycetes are not clearly understood. They were traditionally described as second class (deutero-) members of the "Perfect" sexually-reproducing organisms. At one time, asexual ascomycetes and basidiomycetes were classified separately in a distinct taxonomic group of the phylum Deuteromycota.

Bruns *et al.* (1991) [ 32 ] asked the question: "If all fungi can be compared through their nucleic acids and placed on a single phylogenetic tree, do we need to maintain the Deuteromycota?" The answer from most mycologists is "No". However, our current poor understanding of fungi and especially their life-cycles means that it will be necessary to consider the mitosporic state as a separate taxonomic entity for the foreseeable future (Hywel-Jones, pers. comm.).

Because of their competitive ability and adaptability Ascomycetes have evolved many forms. In the biology of ascomycetes, the most confusing phenomenon is pleomorphism. This is the ability of a species to generate both asexual and sexual means of reproduction. Teleomorphs are found often much later in the life cycle, so that both teleomorph and anamorph states may be split up temporally by as much as a

year [ 33 ]. Work by Hywel-Jones on insect pathogenic Clavicipitales has demonstrated thus [ 34 , 35 , 36 ].

## 2.3 Form-genus *Aschersonia*

The genus *Aschersonia* was established by Montagne for two phyllogenous fungi reported from the tropics. Saccardo (1878) established the genus *Hypocrella* and it was Massee (1896) that recognised the link between these two genera. The genus *Aschersonia* comprises species which occur superficially on the leaves and stems of living plants (Fig.1). Petch (1921) described this as follows:-

> Stroma cushion - or cup-shaped, mostly brightly coloured with localised spots showing conidial masses, containing pycnidia with conidiophores and paraphysis; conidiophores slender, branched, consisting of thin-walled, mostly awl-shaped phialides; conidia hyaline, one-celled, mostly fusiform, smooth-walled. Teleomorph *Hypocrella* this species co-generic with those which are now included in the genus *Hypocrella* were assigned by the earlier mycologists to *Hypocrea.*

Sometimes the two states are found together with conidia [ 27 , 37, 38 ]. Saccardo in 1878 split off the genus *Hypocrella* from *Hypocrea* Fr. and placed in it the following four species:

- *Hypocrella discoidea* B. & Br.,
- *Hypocrella bambusae* B. & Br.,
- *Hypocrella atramentosa* B. & C., and
- *Hypocrella semiamplexa* Berk.

Of these species, only *H. discoidea* is recognised as a true *Hypocrella* and is now regarded as the type species of the genus [ 38 ]. Of the other three species that Saccardo placed in *Hypocrella*, *H. atramentosa* is the type species of the genus *Dothichlo* G.F. Atk. While the other two were referred to the genus *Balansia* Speg. [ 27 ].

Species of the genus *Aschersonia* are related to the teleomorph state *Hypocrella*. The apparent relationship of *Hypocrella* to *Aschersonia* was first made by Massee (1896) in an account of "*Hypocrella oxyspora*". In the absence of any indication from pure culture study, the discovery of both stages in the same stroma was regarded as the only valid proof of the connection between *Aschersonia* and *Hypocrella*. The early separation of ascospores into fragmented part-spores and the consequent disappearance of the ascus leaving the broken-up spores free in the perithecia, led Berkeley into the mistake of placing the present species in the genus *Aschersonia*. Furthermore, the conidia on the covering of young stromata are similar to the cells of the broken-up ascospores in the current species.

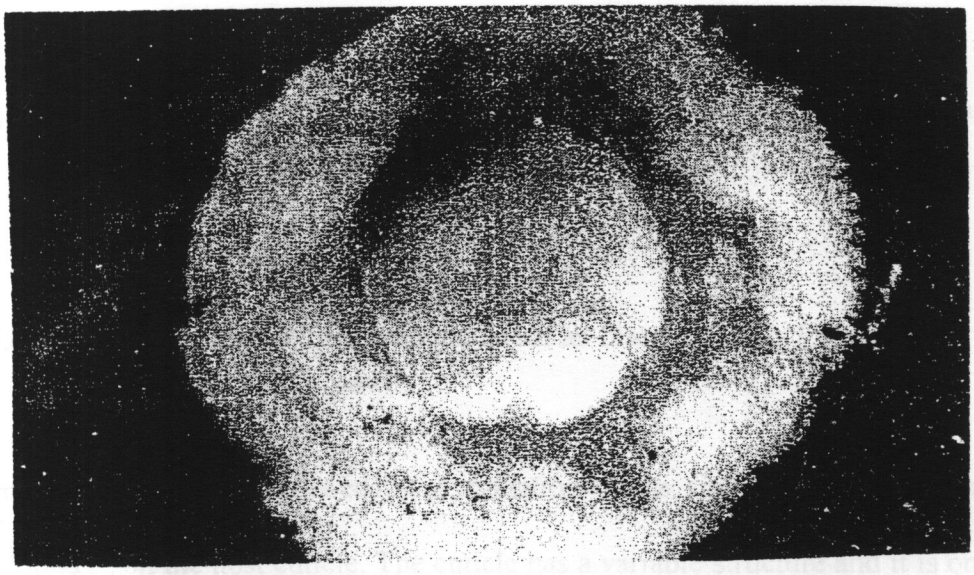

Fig. 2.1 *Aschersonia placenta* covering a scale insect on a bamboo leaf.

## 2.4 Forest habitat

*Aschersonia* spp. are found on scale insects (Lecaniidae) colonising understorey shrubs and trees in tropical regions [ 27 , 37 ]. They can also be collected in the litter layer on leaves fallen from upperstorey trees [ 27 ]. Although on the forest floor it is assumed such specimens play no further role in the life-cycle of the fungus (Hywel-Jones, unpubl. obs.).

## 2.5 Fungus pathogenesis

Entomopathogenic fungi are similar to most fungal pathogens of plants and vertebrates and infect their host through the cuticle [ 39 ]. Commonly entomopathogenic fungi infect the host by penetration through the body wall [ 40 ] while viruses and bacteria infect through the alimentary canal [ 41 ]. Some

entomopathogenic fungi have been shown to infect larvae between the midgut and hindgut [ 42 ].

Three phases are recognized in the development of an insect mycosis [ 43 ]

1. Contact and adhesion, followed by germination of the spore and appressorium development on the host cuticle. The cuticle has a variable structure and it is only possible to give a general description. Four or five layers are recognised by entomologists [ 44 ].

    - an innermost cuticulin layer,
    - a very thin paraffin layer,
    - a protein-polyphenol layer,
    - a wax layer,
    - an outermost cement layer

When washed, conidia of *Beauveria bassiana* (Bals.) Vuill. germinate and grow over the surface of the corn earworm, *Heliothis zea* [ 45 ].This suggests, but does not prove, that the nutrients required for growth of the fungus are secreted and normally available on the larval surface. Growth of *B. bassiana* on autoclaved chitin, with induction of chitinase, occurs because chitin usually contains small inducer molecules that are released into the medium by the autoclaving process.

Successful germination on the host cuticle, however, does not always automatically lead to infection or death.

2. Following germination of the spore, the germ tube attacks the cuticle. Initiation of the infection by most insect fungi is usually through the cuticle [ 44 , 46 ]. Spores have been seen to achieve this by a combination of mechanical and enzymatic action [ 26 ]. Entomopathogenic fungi produce a variety of endo- and exo- acting proteolytic enzymes in culture [ 41 ]. Testing purified enzymes against locust cuticle *in vitro*, St. Leger *et al.*, [ 47 , 48 ] showed that pre-treatment or combined treatment with endo-protease was necessary for high chitinase activity. Samsinakova *et al.*, (1971) [ 49 ] and Smith *et al.*, (1981) [ 50 ] using semipure commercial enzyme preparations against cuticles of *Galleria mellonella* and *Heliothis zea* larvae, respectively concluded that cuticular chitin is shielded by protein.

3. Progression of the fungus inside the insect body usually (but not always) results in the death of the infected host [ 43 ] (Fig. 2.2).

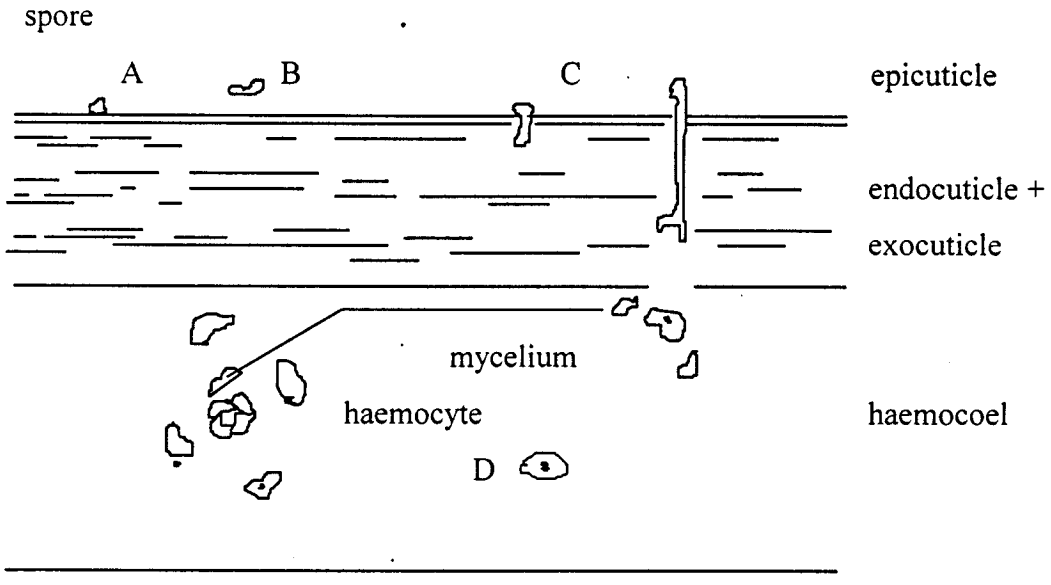


Fig. 2.2 Establishment of a mycosis in an invertebrate. A) Adhesion of the spore to the cuticle, B) germination and development of an appressorium, C) penetration of the cuticle by the germ tube, D) colonisation of the haemocoel and host defence reaction.

Among the three phases recognized in the establishment of a fungal infection in an arthropod host, the first two phases: the adhesion and germination of the fungal spore and the penetration of the cuticle by the germ tube, appear the most critical to the success of the infection. Usually, the multicellular defence reaction of the haemocytes and/or the humoral reaction [ 51 ] if present, seem effective only against weak pathogens or very low doses of aggressive pathogens because fungi have developed several mechanisms to escape the insect defence reaction. Several species of Entomophthorales can spontaneously form protoplasts in the haemocoel of the insect, and are able to grow *in vivo* and *in vitro* in this protoplast stage [ 52 , 53 ]. It has been shown, for example, that fast-growing saprobes e.g. *Aspergillus, Fusarium*

and *Mucor* can easily infect an insect after direct injection into the haemocoel or after a wound in the cuticle [ 54 ]. Immune reactions occurring in invertebrates may help to clear up the phylogeny of the more complex mammalian immunological system. For example, many infectious diseases, such as malaria, are transmitted by insects and the causal organisms develop and migrate into their host insect without developing any cell reaction [ 55 ].

## 2.6 Reproduction and Spores

One of the interesting aspects of fungal biology is the diversity of life cycle patterns found within the major groups. The ability to produce several types of spores gives a fungus advantages to adapt in scattering and survival during suboptimal environmental conditions. This is an important ability for organisms that are essentially sessile in their somatic state. Spores may be produced at different times in the life of a single organism in response to environmental conditions, vary in active expense and survival time, and in some cases, even have different scattering agents [ 33 , 34 , 35 , 36 ].

**Conidia:** Most mitosporic fungi reproduce by means of special spores known as conidia (singular: conidium). A conidium is a non-motile, asexual spore formed at the tip or side of a sporogenous cell. It should be noted that conidia are not produced as a result of growing cleavage of cytoplasm (as in the formation of sporangiospores); thus, they are not surrounded by an additional sporangial wall. They often have great dispersability, but viability of shorter duration than spores

produced in sexual reproduction. Conidia germinate by germ tube development to produce a widespread mycelium and, following that, conidia again. This procedure is known as macrocyclic conidiation [ 56 ]. Furthermore, some fungi may germinate by producing conidia directly from an ascospore or conidium; yeast-like buds or microconidia distinct in morphology from other conidia in the life-cycle of the fungus may be produced as well. This is microcyclic conidiation, which produces new conidia very quickly. In most cases, the dispersal strategies are different with macrocyclic conidia being dispersed away from the parent hyphae and microcyclic conidia staying near the site of their production [ 57 , 58 ].

In some species conidia accumulate, after formation, in a wet droplet which may be water- or animal-dispersed; others are produced in dry masses, and these spores are dispersed efficiently by wind. Dispersal of conidia parallels that of sexual spores; conidium release differs from the active process for sexual spores in most ascomycetes and basidiomycetes because it is usually an inactive (passive) procedure. For example *Aschersonia aleyrodis*, produces slimy masses of conidia in conidiomata on colonized insects [ 59 ]. These conidia are readily splash-dispersed by rainfall. However, species such as *Zygosporium*, an ascomycete, and a number of basidiomycetes [ 60 ] have conidia that are discharged forcibly.

There is an enormous variety of morphologically different types of conidia produced by mitosporic fungi. Their shape may be spherical, ovoid, elongate, cylindric, thread-like, spirally curved, or branched. They may be one- to many-celled, with either cross septa or both transverse and longitudinal septa (a condition known as muriform). Conidia may be hyaline or coloured in shades of yellow, pink, green, brown or black.

These characters of shape, colour and number and order of cells were combined by Saccardo, into the Saccardoan Classification system with informal groups defined on the basis of combinations of these characters. For example, Phragmosporae was described by Saccardo as a group with oblong conidia having two to many cross septa and could be divided further on the basis of colour:

- Hyalophragmiae with hyaline conidia, and
- Phaeophragmiae with pigmented conidia.

The conidia are produced in conidiomata. The phases of conidium development helps to understand the subsequent definitions [ 61 , 62 ].

1. **Initiation:** Conidia may be initiated from a hypha either by conversion of a pre-existing hyphal piece: thallic (arthroconidia) or blown out *de novo*: blastic (blastoconidia).
2. **Wall formation:** The wall of the conidium may be in continuous connection with the entire wall of the conidiogenous cell or at least with some layers of it or may be formed *de novo*. If both wall layers of the conidiogenous cell and the conidium are continuous the process is hologenous. If the outer wall of the conidium is continuous with only the inner wall layer of the conidiogenous cell this is known as enterogenous.

3. **Conidium secession:** Conidia secede when they become detached from the conidiogenous cell. The mature conidium secedes either by cleavage at a separating septum or by fracture of the lateral wall between two septa. There are two methods of secession:

- **Schizolytic** secession occurs when two wall layers of the delimiting septum separate.
- **Rhexolytic** secession is when the entire septum separates with the conidium often tearing the cell directly below. However, the cell beneath the conidium may be a specialised separating cell that breaks or collapses.

## 2.7 Some benefits of *Aschersonia*

### 2.7.1 *Aschersonia* as agents of biological control

In recent years there has been an increase in environmental pollution through the use of chemical biocides to control pest organisms such as insects, weeds and fungi that threaten human interests. These chemical biocides affect mankind resulting in abnormalities or harmful diseases such as cancer. However, many years ago before chemicals became widely accepted fungi were considered as biological biocides and were successful for the control of many insect pests [ 63 , 64 ]. Although widely used in the late nineteenth and early twentieth century fungi became less accepted as

cheaper and more reliable chemicals became available. Under natural conditions the fungus populations may build up to very high levels, but not quickly enough to control the target pest insect during the period when it causes the most damage. Hywel-Jones (pers. comm.) has noted that many fungal pathogens of agricultural pests seem to appear only when the crop is mature and insects are suffering from physiological stress due to crowding and/or reduction in the palatability of the crop. Fungi, however, have several potential shortcomings as biological control agents.

- They may only damage, rather than kill, their host.
- They may only reduce, rather than eliminate, the target population.
- They may do both of these things relatively slowly.

These outcomes are not entirely satisfactory to farmers, who are used to the quick action and high kill-rate of chemical biocides. Several species of insect fungi cause spectacular epidemics in natural insect populations and are now being grown in large-scale artificial culture to produce inoculum with the hope of inducing epidemics on demand [ 65 , 66 ]. Four hyphomycetes have proved so successful that spray concentrates containing their spores are now sold under trade names as myco-insecticides (Table 1).

2.7.2 *Aschersonia* as sources of metabolites

⇒ Chymoelastases and trypsin-like enzymes from *Aschersonia*

*Aschersonia aleyrodis* produced basic (pI greater than 7.0) chymoelastases with multiple binding sites, comprising at least four or five subsites, with preference for hydrophobic residues at the primary binding site [ 26 ]. Most isolates also produced additional acidic enzymes with similar specificities against ester and amide substrates but which lacked activity against elastin. Both acidic and basic enzymes degraded high protein azure or locust cuticle and, as shown by inhibition studies, possessed essential serine and histidine residues in the active site [ 26 ].

Table 2.1 Some commercially produced fungal pathogens of arthropods.

| Genus | Trade name | Phylum | Principle target |
|---|---|---|---|
| *Coelomomyces* | | Chytridiomycota | Mosquito larvae |
| *Entomophthora* | | Zygomycota | Aphids |
| *Conidiobolus* | | Zygomycota | Aphids |
| *Beauveria* | **"Boverin"** | Dikaryomycota (Hyphomycetes) | Colorado beetle, Codling moth |
| *Hirsutella* | **"Mycar"** | Dikaryomycota (Hyphomycetes) | Citrus rust mite |
| *Metarhizium* | **"Metaquino"** | Dikaryomycota (Hyphomycetes) | Spittlebug lavae Mosquito, Rhinoceros beetle, Lepidopteran larvae |
| *Verticillium* | **"Vertalec"** | Dikaryomycota (Hyphomycetes) | Aphids |
| *Verticillium* | **"Mycotal"** | Dikaryomycota (Hyphomycetes) | Whitefly |
| *Verticillium* | **"Thriptal"** | Dikaryomycota (Hyphomycetes) | Thrips |
| *Nomuraea* | | Dikaryomycota (Hyphomycetes) | Lepidopteran larvae |
| ***Aschersonia*** | | Dikaryomycota (Hyphomycetes) | Whitefly, scale insects |

⇒ *Aschersonia* as a source of carotenoids

Carotenoids represent one of the most widely distributed and structurally diverse classes of natural pigments, with important functions in photosynthesis, nutrition and protection against photo-oxidative damage [ 67 ]. This wide range of properties make them an important and complex class of biological pigments. Many eucaryotes, including all algae and plants, as well as some fungi, also synthesise these pigments. *Aschersonia aleyrodis* contains six carotenes with beta-carotene (87%) as the major pigment [ 25 ]. In old cultures there is a decrease in total carotenoids, the disappearance of two trans-carotenes and the appearance of two cis-carotenes [ 25 ].

2.8 Taxonomy of *Aschersonia* used in this study

***Aschersonia hypocreoidea* (Cooke & Massee) Petch, Ann. R. Bot. Gdns, Perad VII (1921) p. 255.**

= *Fusarium hypocreoideum* Cooke & Massee, Grevillea, XVI, (1888). p. 76.

= *Aschersonia zenkeri* P. Henn., Engler's Bot. Jahrb., XXIII. (1897), p. 541.

Stromata circular or oval, up to 3 mm diameter, almost plane, about 250 μm thick, tomentose or byssoid, sometimes surrounded by a thin, membranous or powdery hypothallus, white, rarely yellow. Conidiomata in the centre of the stroma, arranged irregularly or in a circle, concave, widely open, sometimes somewhat flask-shaped and convoluted at the base; spore masses pale yellow or orange yellow, distinct or

confluent; conidia narrow-oval or lanceolate, ends pointed but not produced 8-13 x 2-2.5 μm; paraphyses up to 120 μm long. The *Aschersonia hypocreoidea* two strains in this study show conidia phialides and paraphyses (Fig. 2.3a-2.4b).

**Known distribution:** Australia, Indonesia, Philippines, Sri Lanka, Taiwan (Formosa), Thailand, Vietnam (Tonkin) and West Africa [ 27 , Hywel-Jones, unpubl. obs. ].

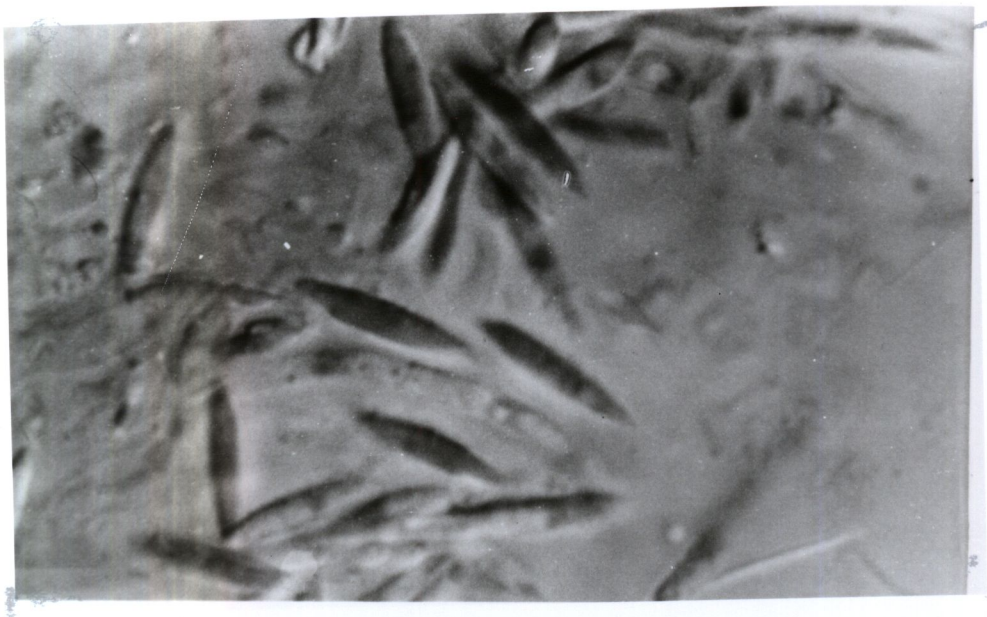

Fig. 2.3a Conidia of *A. hypocreoidea*-3162 grown on PDA at 22°C.

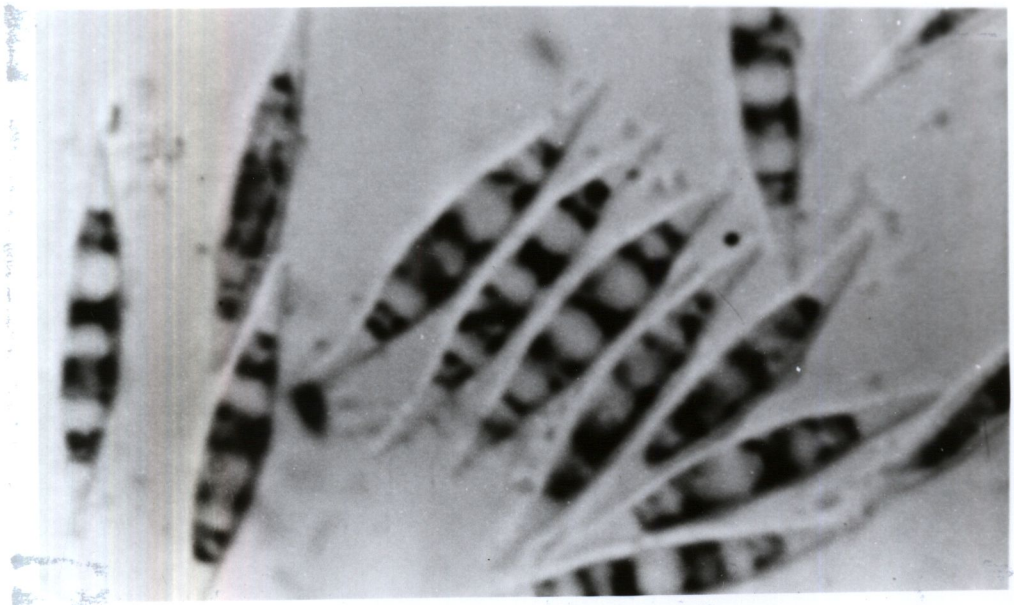

Fig. 2.3b Conidia of *A. hypocreoidea*-5269 grown on PDA at 22°C.

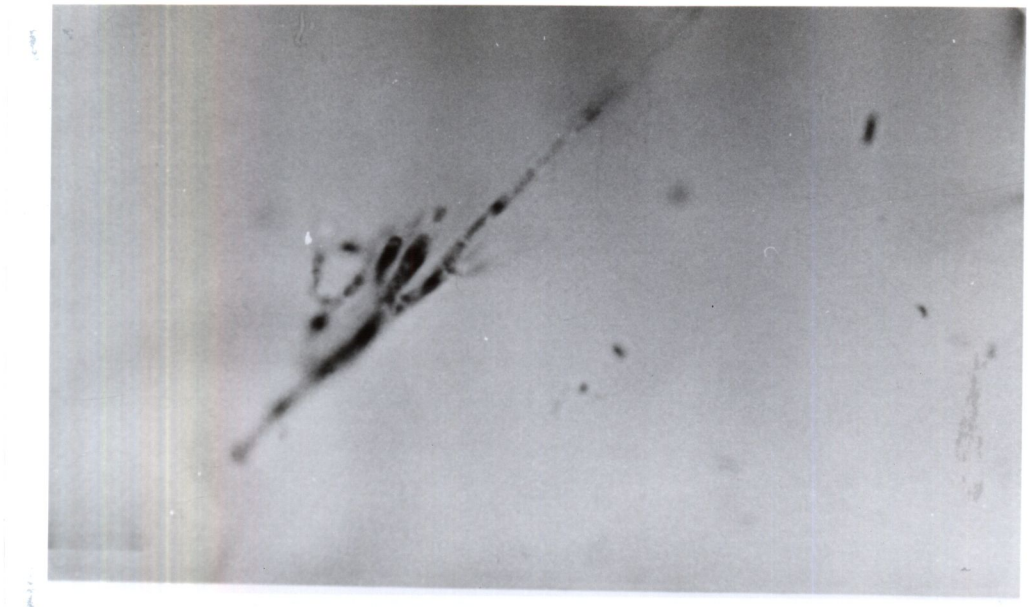

Fig. 2.4a Phialides and paraphyses of *A. hypocreoidea*-3162 grown on PDA at 22°C.

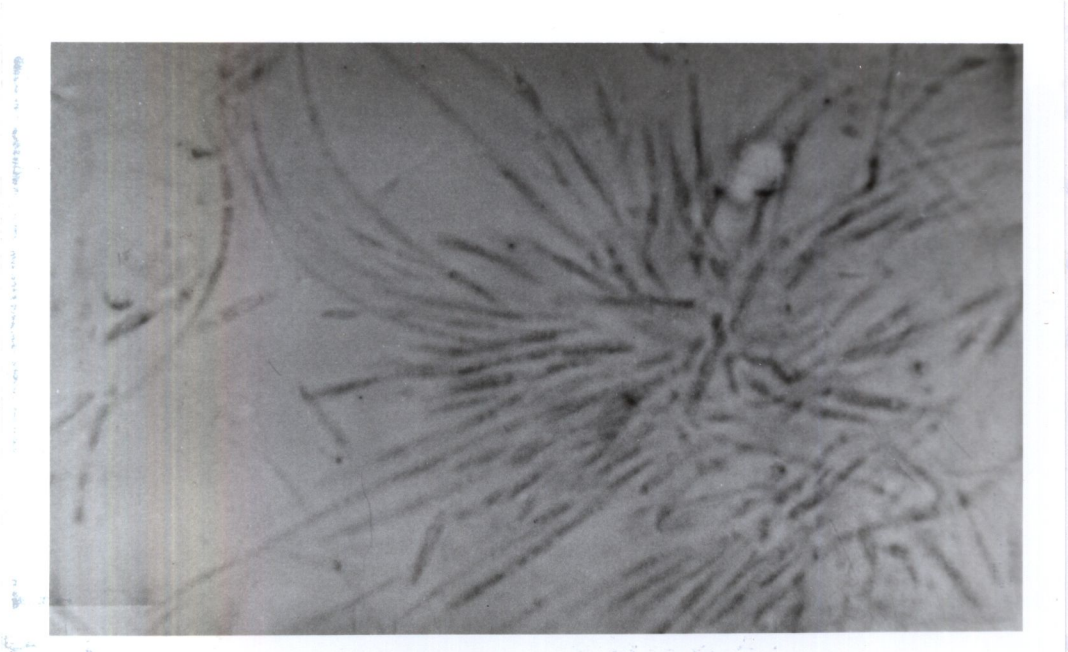

Fig. 2.4b Phialides and paraphyses of *A. hypocreoidea*-5269 grown on PDA at 22°C.

***Aschersonia placenta* B. & Br., Jour. Linn. Soc., XIV, (1873) p. 89.**

= *Aschersonia novoguineensis* P. Henn., Engler's Bot. Jahrb., XXV. (1898), p. 509.

= *Aschersonia javanica* Penz. & Sacc., Malpighia (1901), p. 236.

= *Aschersonia lecanioides* P. Henn., Hedwigia (1902), p. 145.

Stromata various, usually widely open, irregular, globose or lenticular, lobed cavities, up to 400 x 300 μm sometimes flask-shaped with a well-defined neck, up to 600 μm deep and 250 μm diameter. Conidiomata openings in the flatter forms usually circularly arranged and radially elongated; in the pulvinate forms scattered and circular. Conidia extruded in red or red brown masses, fusoid, ends acute, 10-14 x 1.5-2 μm: paraphyses linear, 40-80 μm long. Ascigerous stroma developing from the conidiomatal stroma, rarely distinct, consisting of globose, ovoid or cylindric processes, arising round the margin of the flatter stroma, or anywhere on the pulvinate stromata. The *Aschersonia placenta* strain in this study show conidia phialides and paraphyses (Fig. 2.5a-2.5b).

**Known distribution:** Africa, India, Indonesia, Malaysia, Papua New Guinea, Philippines Singapore, Sri Lanka and Thailand [ 27 , Hywel-Jones, unpubl. obs. ].

***Aschersonia* state of *Hypocrella tubulata* Petch.**

Ascomatal stromata white, minutely tomentose, varying from pulvinate, 1.6 mm diameter, 1 mm high, slightly tuberculate, or strongly tuberculate with rounded tubercles, 400 μm diameter, to flattened pulvinate, 2 mm diameter, 600 μm high, strongly tuberculate and irregularly divided; usually with a fibrillose or scarious hypothallus which may be 1 mm wide in the flatter irregular forms; ostiola slightly elevated, yellow, translucent; perithecia from subglobose, 400 μm diameter, to narrow flask-shaped, 200-400 μm diameter, 400-700 μm high, embedded singly in the tubercles in the flatter forms, and in the stroma in the pulvinate forms in the latter case opening through the tubercles if present; asci up to 200 x 8-10 μm; part-spores cylindric with rounded ends or narrow-oval, 7-12 x 2-2.5 μm.

***Aschersonia*** state: stromata sometimes pulvinate and even, usually a pulvinate or flattened disc up to 2 mm diameter, from which arise columnar processes, either abruptly or gradually; processes conical or cylindric, up to 600 μm high, 400 μm diameter, truncate at the apex, with a wide ostiolum which is often laterally compressed; stroma white minutely tomentose, with a tomentose or fimbriate margin, becoming more compact and subglabrous when old; tissue of the columns composed of more or less parallel hyphae; conidiomata cylindric, globose, or lenticular, sometimes convoluted at the base, usually with a long cylindrical neck conidia fusoid, ends blunt, 8-10 x 1.5 μm paraphyses linear, up to 150 μm long. The conidiomatal stromata are very variable, but in general they may be identified by the

long cylindrical neck of the pycnidium. The *Aschersonia tubulata* two strains in this study show conidia phialides and paraphyses (Fig. 2.6a-2.7b).

**Known distribution:** Sri Lanka and Thailand [ 27 , Hywel-Jones, unpubl. obs. ].

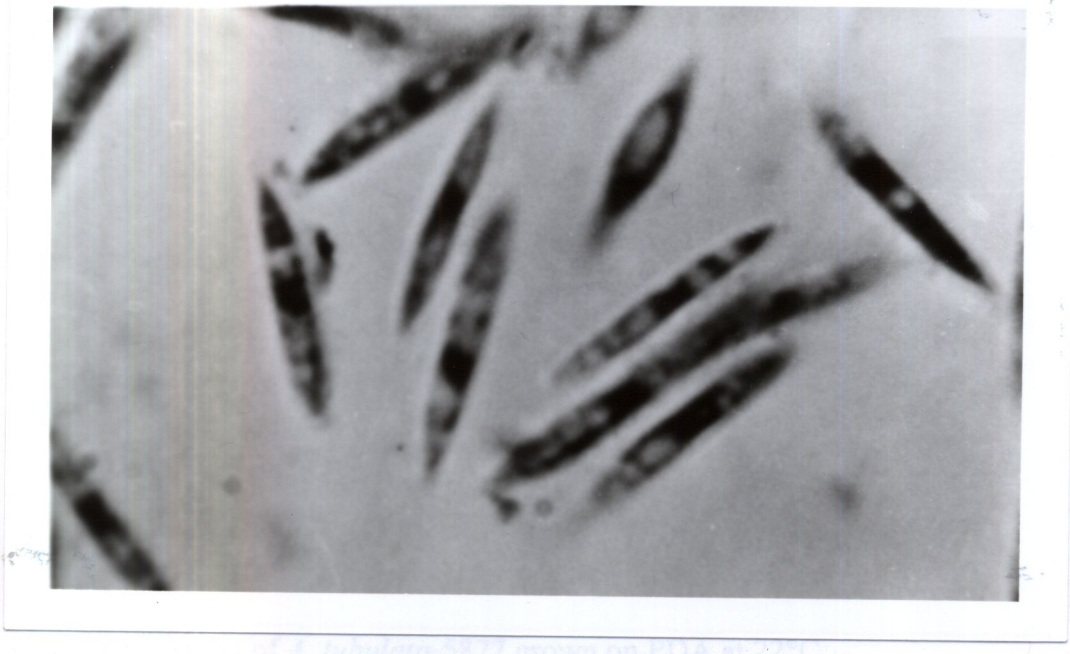

Fig. 2.5a Conidia of *A. placenta*-5164 grown on PDA at 22°C.

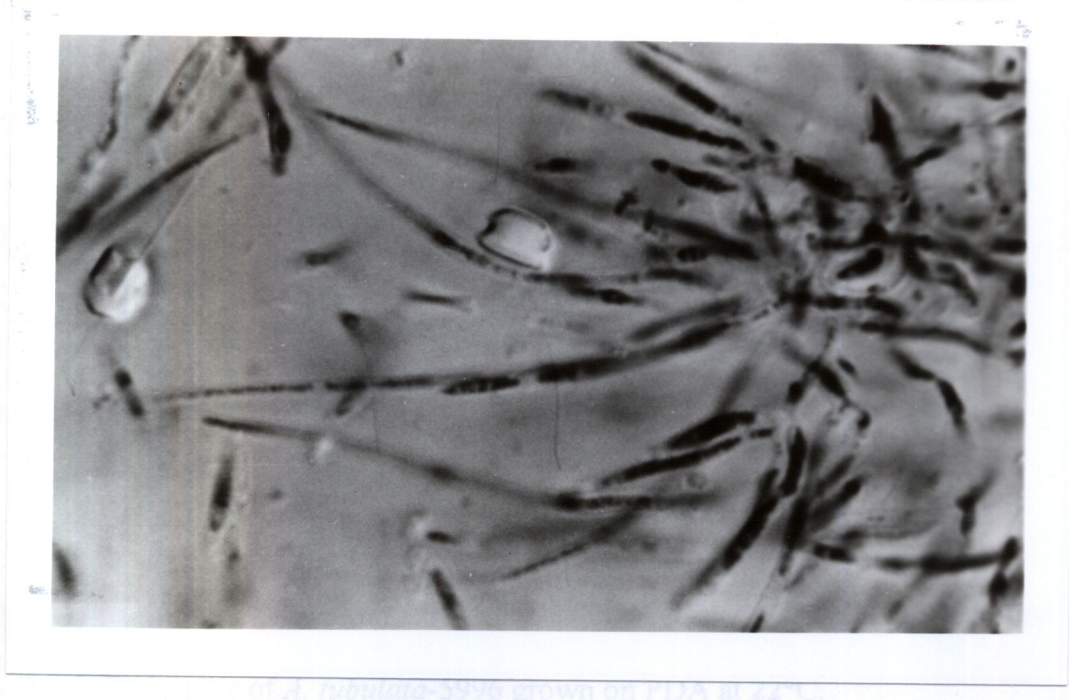

Fig. 2.5b Phialides and paraphyses of *A. placenta*-5164 grown on PDA at 22°C.

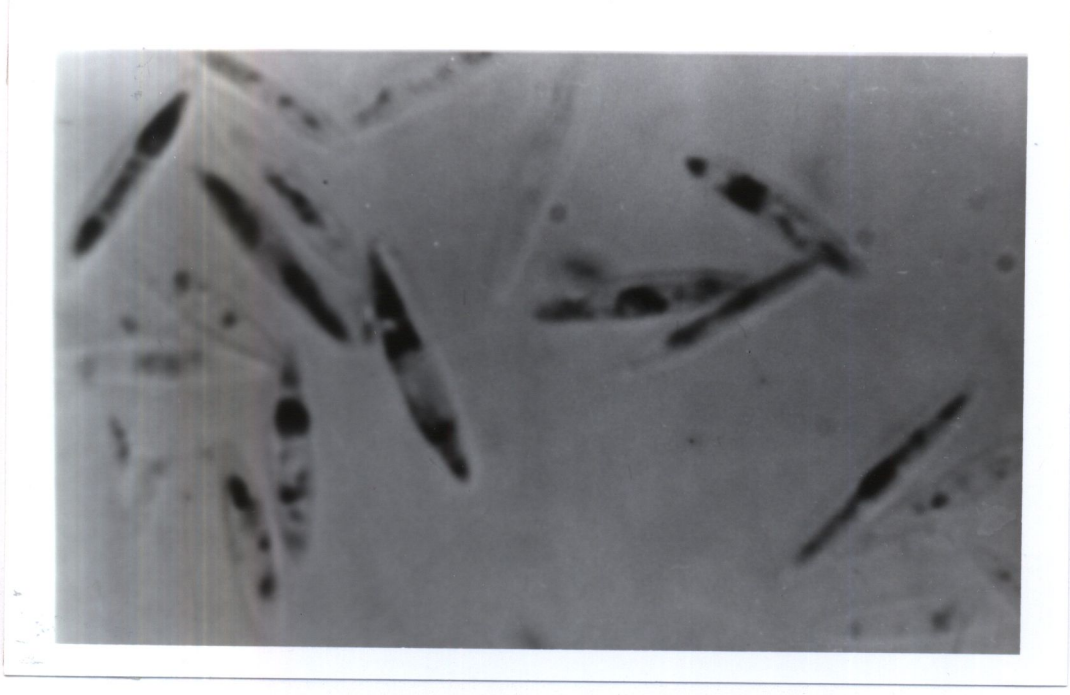

Fig. 2.6a Conidia of *A. tubulata*-5877 grown on PDA at 22°C.

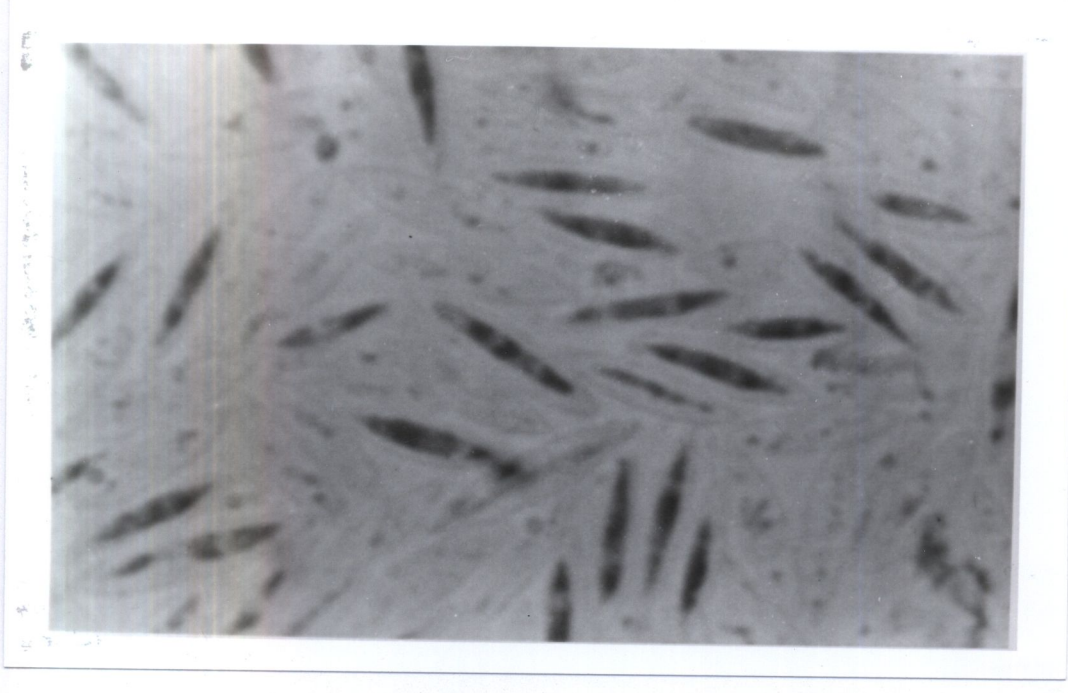

Fig. 2.6b Conidia of *A. tubulata*-5996 grown on PDA at 22°C.

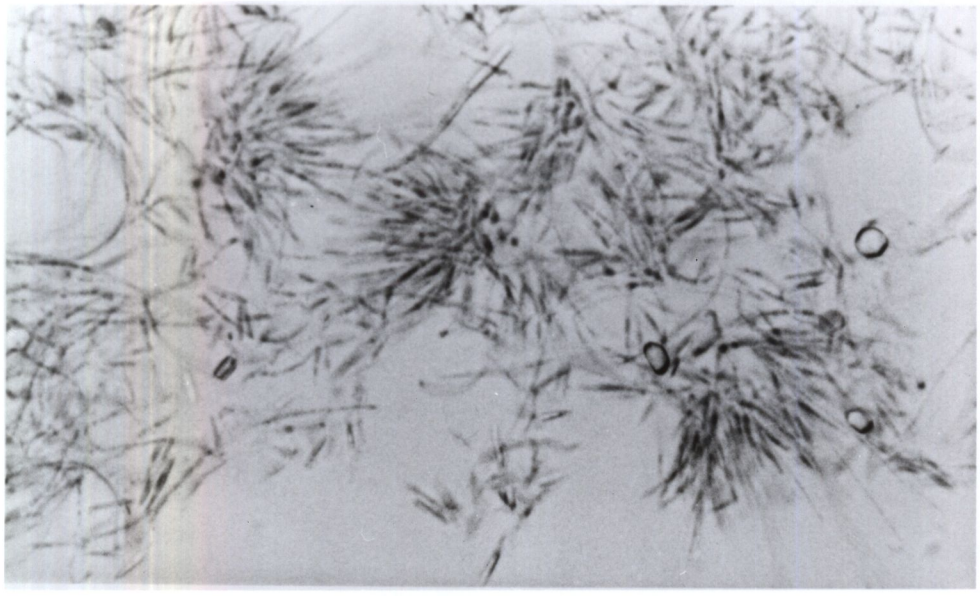

Fig. 2.7a Phialides and paraphyses of *A. tubulata*-5877 grown on PDA at 22°C.

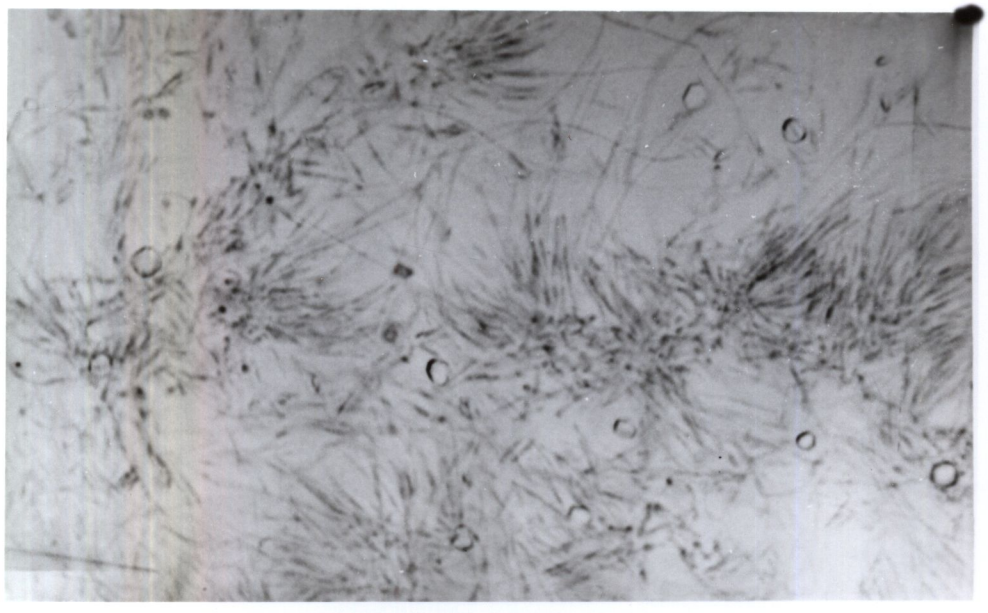

Fig. 2.7b Phialides and paraphyses of *A. tubulata*-5996 grown on PDA at 22°C.

# Chapter 3

## Theory: The fatty acids

### 3.1 Fatty acid nomenclature

Fatty acids have been named in several different ways. As with the nomenclature of fungal structures, fatty acid nomenclature has changed with time. Many unrelated names are still used and have accordingly to be known. These names were initially given before the chemical structure of fatty acids was resolved and were often chosen to indicate the source of the acid. Examples include:

- palmitic (from palm oil),
- oleic (from olive oil, *Olea eupopea*),
- linoleic and linolenic (from linseed oil),
- ricinoleic (from castor oil, *Ricinus communis*)
- arachidonic acid (from groundnut oil, *Arachis hypogea*).

Occasionally the name was linked with the scientist who first described the acid or some significant property of that acid, e.g. Mead's acid (20:3 *n*-3). Sometimes an acid had more than one name until the unity of the material was resolved. Thus, 9-hexadecenoic acid was earlier designated palmitoleic acid and also zoomaric acid [ 68 ]. A trivial name may continue to be used because the systematic name is

*unmanageable as for example with α-elostearic acid which is simpler than 9c11t13t-* octadecatrienoic acid. Trivial names are easy to use but they are not, in themselves, indicators of structure. Systematic names are based on internationally acclaimed rules agreed by organic chemists and biochemists. Those who know the rules can interconvert systematic names and structure.

## 3.2 Main structural features

The number of known natural fatty acids exceeds 1000 although only a small number (perhaps 20-50) are of common concern [ 68 ]. Based on a survey of these 1000 structures, and noting especially the structures of those acids produced most commonly in nature, it is possible to make four basic statements. Each of these is ordinarily true but there are exceptions to all four. The exceptions are frequently trivial but sometimes they are important. Though originally based on a survey of chemical structures it is now clear that these statements also reflect the underlying biosynthetic pathways by which the acids are produced in nature.

(i) Natural fatty acids - straight chain compounds both saturated and unsaturated - are with an even number of carbon atoms in their molecules. This is true for the large majority of structures and for the more abundant acids. Chain lengths range from two to over 80 carbon atoms although they are most commonly between $C_{12}$ and $C_{22}$. In spite of the validity of this statement, fatty acids with an odd number of carbon atoms (e.g.

heptadecanoic, $C_{17}$) occur as do those with branched chains (e.g. isopalmitic, anteisononadecanoic) or with carbocyclic units (e.g. sterculic, chaulmoogric).

(ii) Acids with one unsaturated centre are generally olefinic compounds with *cis* (Z) order and with the double bond in one of a limited number of preferred positions. This is most commonly ∝-9 (i.e. nine carbon atoms from the carboxyl group as in oleic) or *n*-9 (i.e. nine carbon atoms from the methyl group as in oleic or erucic acid). But double bonds occur in other positions (e.g. petroselinic, 6*c*-18:1), or have *trans* configuration (e.g. elaidic 9-*t*-18:1), or can be replaced by an acetylenic unit (e.g. tariric 6*a*-18:1).

(iii) Poly unsaturated acids are mainly polyolefinic with a methylene-interrupted order of double bonds having *cis* (Z) configuration. That is, *cis* double bonds are separated from each other by one $CH_2$ group as in arachidonic acid:


The 1,4-pattern of unsaturation is characteristic of natural fatty acids and differs from that in acyclic isoprenoids which is usually 1,3 (conjugated) or 1,5 polyunsaturated acids occur in biosynthetically related families. The most

important are the *n*-6 acids based on linoleic and the *n*-3 acids based on ∝-linolenic acids. In contrast to this very common pattern of unsaturation some fatty acids have conjugated unsaturation which may be *cis* or *trans* (e.g. eleostearic, calendic and parinaric acids), some have mixed en/yne unsaturation which may be conjugated (e.g. isanic) or non-conjugated (e.g. crepenynic), and some have non-conjugated unsaturation which is not entirely methylene-interrupted. These are known as non-methylene interrupted polyenes (e.g. columbinic and pinolenic). Fatty acids rarely have functional groups apart from the carboxyl group and the various types of unsaturation already discussed. However, acids are known which also contain a fluoro-, hydroxy-, keto- or epoxy- group. Two important examples are ricinoleic (12-hydroxyoleic acid) and vernolic acid (12,13-epoxyoleic).

## 3.3 Isolation and identification

Indications that an unusual acid is present in a sample may be apparent from unexpected behaviour during routine chromatographic or spectroscopic examination. It is then necessary to isolate or concentrate the unknown acid prior to categorizing it further. The structure can usually be defined by comparison with an authentic sample or with compounds of related

structure if these are already known. The gas chromatography (GC) can be used to compare the retention time of unknown fatty acids with known standards. When a

fatty acid has a completely novel structure then further investigation is required and spectroscopic procedures are likely to provide the information to help determine structure. Chromatographic procedures of separation are now often combined with spectroscopic methods of identification in a single operation. These are described as 'hyphenated techniques' and include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-gas chromatography (LG-GC), liquid chromatography-Fourier transform infrared spectroscopy (LC-FTIR), etc. To fully define the structure of a fatty acid it is necessary to know:

1. Its chain length and whether the molecule contains any branched or cyclic systems or any of the less common functional groups;
2. The number, nature, configuration and position of all unsaturated centres;
3. The nature, position and (where relevant) the stereochemical configuration of any functional groups.

The classical partition procedures of condensation and crystallisation are only of limited value to lipid chemists. With polyunsaturated acids it is desirable to avoid the high temperatures associated with fractional distillation since these promote undesirable changes such as stereomutation, double bond migration, cyclization and dimerization. Distillation allows separation by chain length and is used industrially for this purpose. It is of only limited value for separating acids/esters differing only in their degree of unsaturation. Limited use is still made of urea fractionation.

Saturated fatty acids form stable complexes more readily than do the unsaturated fatty acids. In practice urea and mixed acids are dissolved in hot methanol or urea and methyl esters in a hot methanol-ethanol mixture. The solution is crystallised at room temperature or at 0°C. The adduct and mother liquor will furnish the acids or esters when mixed with water and extracted with ether or petroleum ether. The procedure is used for two purposes. It separates straight-

chain acids or esters from branched-chain or cyclic compounds with the former concentrating in the adduct and the latter in the mother liquor. Urea fractionation is also used to separate acids or esters of differing unsaturation.

Many fatty acids differ so little in solubility that they can rarely be purified by crystallization. Most useful separations are now achieved by chromatography, some of which can generally provide sufficient material for subsequent spectroscopic examination. Table 2 shows some fatty acids (saturated and unsaturated fatty acids) that are found in nature [ 69 ].

Table 3.1 Some fatty acids that are found in nature.

a) Saturated fatty acids

| Symbol | Common name | Systematic name | Structure | melting point (°C) |
|---|---|---|---|---|
| 12:0 | Lauric acid | Dodecanoic acid | $CH_2$-$(CH_2)_{10}COOH$ | 44.2 |
| 14:0 | Myristic acid | Tetradecanoic acid | $CH_3$-$(CH_2)_{12}COOH$ | 52 |
| 16:0 | Palmitic acid | Hexadecanoic acid | $CH_3$-$(CH_2)_{14}COOH$ | 63.1 |
| 18:0 | Stearic acid | Octadecanoic acid | $CH_3$-$(CH_2)_{16}COOH$ | 69.6 |
| 20:0 | Arachidic acid | Eicosanoic acid | $CH_3$-$(CH_2)_{18}COOH$ | 75.4 |
| 22:0 | Behenic acid | Docosanoic acid | $CH_3$-$(CH_2)_{20}COOH$ | 81 |
| 24:0 | Linoceric acid | Tetracosanoic acid | $CH_2$-$(CH_2)_{22}COOH$ | 84.2 |

b) Unsaturated fatty acids

| (all double bonds are cis) | Common name | Systematic name | Structure | melting point (°C) |
|---|---|---|---|---|
| 16:1 | Palmitic acid | 9-Hexadecanoic acid | $CH_3$-$(CH_2)_5$-H=CH$(CH_2)_7$COOH | -0.5 |
| 18:1 | Oleic acid | 9-Octadecanoic acid | $CH_3$-$(CH_2)_7$-H=CH$(CH_2)_7$COOH | 13.4 |
| 18:2 | Linoleic acid | 9,12- | $CH_3$-$(CH_2)_4(CH{=}CHCH_2)_2$ | -9 |

| | | | | |
|---|---|---|---|---|
| | | Octadecadienoic acid | $(CH_2)_6COOH$ | |
| 18:3 | α-Linolenic acid | 9,12,15-Octadecanoic acid | $CH_3CH_2(CH=CHCH_2)_3(CH_2)_6COOH$ | -17 |
| 18:3 | Δ-Linolenic acid | 6,9,12-Octadecatrienoic acid | $CH_3(CH_2)_4(CH=CHCH_2)_3(CH_2)_3COOH$ | -43 |
| 20:4 | Arachidonic acid | 5,8,11,14-Eicosatetraenoic acid | $CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$ | -49.5 |
| 20:5 | EPA | 5,8,11,14,17-Eicosapentaenoic acid | $CH_3CH_2(CH=CHCH_2)_6(CH_2)_2COOH$ | -54 |
| 24:1 | Nervalic acid | 15-Tetracosenoic acid | $CH_3(CH_2)_7CH=CH(CH_2)_{13}COOH$ | 39 |

## 3.4 Gas chromatography

Currently, gas chromatography is the technique most commonly used to separate methyl esters for quantitative analytical purposes. Originally packed columns were used but the more efficient capillary columns are now preferred. The separated ingredients are not generally isolated but a careful study of elution behaviour may indicate chain length, degree of unsaturation and perhaps the position of unsaturated centres. For identification purposes there are some benefits in operating under isothermal conditions. Retention times can be compared with those of an internal

standard which is usually a saturated ester such as palmitate or stearate, or the retention behaviour can be expressed in terms of equivalent chain length (ECL) first suggested by Miwa *et al.*, (1960) [ 70 ]. This is the notional number of carbon atoms in a saturated ester whose methyl ester would co-elute with the ester in question. For example, if methyl oleate has an ECL of 18.50 this means that it would elute between the $C_{18}$ and $C_{19}$ methyl esters at the same time as a saturated ester with 18 1/2 carbon atoms. This concept is based on the observation that for any homologous series eluted under isothermal conditions the plot of log retention time against the number of carbon atoms is linear.

## 3.5 Preparation of Fatty Acid Methyl Esters for Gas-Chromatographic Analysis of Lipids in Biological Material

Theoretically, preparation of fatty acid methyl esters deals with reversible chemical reactions in a complex system.

### 3.5.1 Acid-catalyzed transmethylation/methylation

Lipids are mainly a combination of esters and preparation of fatty acid methyl esters (FAMEs) involves converting one ester to another. The reaction is referred to as "transesterification" in general and "transmethylation" in particular. Because the reaction involves cleavage of an ester by an alcohol, transesterification is also referred to as

"alcoholysis" and transmethylation as "methanolysis." When FAMEs are from interactions between fatty acids and methanol, the reaction is termed "methylation" (esterification). Therefore, strictly speaking methylation and transmethylation (or esterification and transesterification) refer to different reactions although in the literature these terms are used interchangeably.

According to the principles of organic chemistry, both transesterification and esterification are reversible reactions. Importantly transesterification can be catalysed by either an acid or a base:

$$\text{R-CO-OR} + \text{R}'\text{-OH} \xleftrightarrow{H^+ \text{ or } {}^-OCH_3} \text{R}'\text{-CO-OR} + \text{R-OH}$$

Whereas esterification can not be catalysed by a base but can be by an acid:

$$\text{R-CO-OH} + \text{R}'\text{-OH} \xleftrightarrow{H^+} \text{R}'\text{-CO-OR} + \text{HOH}$$

Acid-catalysed transmethylation/methylation: Acidic catalysts not only transesterify triglycerides and other complex lipids but also esterify free fatty acids in the presence of methanol. Three commonly used acid reagents are hydrochloric acid (HCl), sulphuric acid ($H_2SO_4$) and boron flouride ($BF_3$) all in methanol. Heating is required to speed up the reaction. The temperature may range from 60-90°C and duration may last from a few minutes to several hours. For this group of reagents, care should be taken to avoid concentrations higher than recommended otherwise undesirable side

reactions will occur. One apparent cause of these side reactions is the loss of unsaturated esters.

Transesterification of fatty acids with anhydrous HCl/MeOH for gas-liquid chromatography was introduced about forty years ago [ 71 ] It is one of the milder reagents and has been claimed to be the best general purpose esterifying agent [ 72 ]. The

reagent can be prepared either by bubbling anhydrous hydrogen chloride gas into methanol or by adding liquid acetyl chloride slowly to methanol [ 73 ]. The concentration is normally 5%. Limited stability of the reagent was reported by Kishimoto and Radin (1965) [ 74 ] who found that half the titratable acid was lost at room temperature in six weeks. Alternatively, aqueous HCl in methanol (36% HCl solution/MeOH = 4:1 vol/vol) has also been used [ 75 ]. Because of the different methods reported in the literature and because many researchers used or modified a procedure for FAMEs preparation without giving a reference, it is difficult to trace the first reference to direct transmethylation. It is also difficult to find out the primary procedure on which a modification is based. Regardless of these difficulties, and regardless of variations in procedures described the direct transmethylation generally involves adding an organic solvent and a methanolic-catalyst reagent to a small amount of sample preferably dehydrated in a test tube and heating the mixture for a certain time period (10 min to several hours) depending on heating temperature (65-

100°C) and lipid composition. For quantitative analysis an internal standard is commonly added.

3.5.2 Use of an internal standard:

For some transmethylation/methylation reactions particularly those catalysed by acid reagents, it may take many hours to reach near completion. However, using an internal standard quantitative analysis can be assured without requiring the reaction to go to completion [ 76 ] or worrying about losses of esters when more powerful transmethylation conditions are applied [ 77 ]. Here an assumption is that the relative concentration of FAMEs formed at a given time after the reaction is initiated represents the true composition of fatty acids in the system. However, it should be emphasised that for a given reaction system this assumption may be true only after equilibrium is reached.

# Chapter 4

# Materials and methods

## 4.1 Origin of strains

Five strains representing three species of *Aschersonia* were used in this study. These were isolated from various sites in Thailand (Table 3).

Table 4.1 List of *Aschersonia* used in this study.

| Species | NHJ Code. | Location | Date of collection |
|---|---|---|---|
| *Aschersonia hypocreoidea* | 3162 | Khao Sok National Park Bang Po Ta | 26/02/94 |
| *Aschersonia hypocreoidea* | 5269 | Khao Yai National Park Heo Narok | 21/03/95 |
| *Aschersonia tubulata* | 5877 | Ko Charng National Park Than Makok | 22/11/95 |
| *Aschersonia tubulata* | 5996 | Khao Pu, Khao Ya-trail to Tham Pla | 15/01/96 |
| *Aschersonia placenta* | 5164 | Ko Charng National Park Than Ma Yom | 13/12/94 |

- Khao Yai National Park is 150 Km north-east of Bangkok and covers 2000 $km^2$. Elevation is from sea level to 1300 m and the forest types include lowland scrub, mixed deciduous and evergreen forest.
- Ko Charng National Park is a group of islands 300 km east of Bangkok and close to the Khmer border. Thirty kilometres long and eight wide it is the second largest island in Thailand after Phuket. Elevation is from sea level to 740 metres and the forest is of the same type as Khao Yai National Park.

- Khao Pu - Khao Ya National Park covers 695 $km^2$ in Phattalung and Trang provinces in the southern peninsula of Thailand. The forest type is mostly lowland evergreen forest.
- Khao Sok National Park covers 646 $km^2$ and dominated by spectacular forest-covered limestone crags rising to 1000 metres.

## 4.2 Culture methods

The five strains of *Aschersonia* were maintained on slopes of Potato Dextrose Agar before being transferred to five solid media.

### 4.2.1 Solid media

All media were autoclaved at 121°C for 15 minutes. The media used in this study were as follows:

Sabouraud Dextrose Agar (SDA)

| | |
|---|---|
| peptone | 15 g |
| glucose | 20 g |
| agar | 15 g |
| distilled water | 1 litre |

Malt Extract Agar (MEA)

| | |
|---|---|
| malt extract | 20 g |
| peptone | 1 g |
| glucose | 20 g |
| agar | 15 g |
| distilled water | 1 litre |

M 102

| | |
|---|---|
| malt extract | 20 g |
| bacto peptone | 2 g |
| sucrose | 30 g |
| yeast extract | 1 g |
| KCl | 0.5 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| agar | 15 g |
| distilled water | 1 litre |

Minimal Salt Media (MSM)

| | |
|---|---|
| $NH_4NO_3$ | 3 g |
| glucose | 20 g |
| $KH_2PO_4$ | 0.5 g |
| yeast extract | 1 g |
| $NaH_2PO_4$ | 0.5 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| $CaCl_2$ | 0.5 g |
| agar | 15 g |
| distilled water | 1 litre |

Potato Dextrose Agar (PDA) (Difco, Detroit, USA.)

| | |
|---|---|
| potato, infusion form | 200 g |
| bacto dextrose | 20 g |
| bacto agar | 15 g |
| distilled water | 1 litre |

4.2.2 Liquid media

The liquid medium was prepared with 10 ml per flask and autoclaved at 121°C for 15 minutes. All studies were done using static culture. The following media were used.

Sabouraud Dextrose Broth (SDB)

| | |
|---|---|
| Peptone | 10 g |
| Glucose | 20 g |
| Distilled water | 1 litre |

Peptone Yeast Glucose Broth (PYGM)

| | |
|---|---|
| Bacto peptone | 5 g |
| Glucose | 10 g |
| Yeast extract | 20 g |
| $KH_2PO_4$ | 1 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| Distilled water | 1 litre |

Sorbitol Media Broth (SM)

| | |
|---|---|
| Sorbitol | 100 g |
| Glucose | 40 g |
| Yeast extract | 20 g |
| $KH_2PO_4$ | 1 g |
| $MgSO_4.7H_2O$ | 0.3 g |
| Succinic acid | 10 g |
| Distilled water | 1 litre |
| Trace element solution | 5.0 ml |

content of trace element solution

| | |
|---|---|
| citric acid | 5 g |
| $Fe(NH_4)_2(SO_4)_2.6H_2O$ | 10 g |
| $ZnSO_4.7H_2O$ | 5 g |
| $MnSO_4.H_2O$ | 0.05 g |
| $Na_2MoO_4.2H_2O$ | 0.05 g |
| $CuSO_4.5H_2O$ | 0.25 g |
| distilled water | 1 litre |

Minimal Salt Media (MSM)

| | |
|---|---|
| $NH_4NO_3$ | 3 g |
| glucose | 20 g |
| $KH_2PO_4$ | 0.5 g |
| yeast extract | 1 g |
| $NaH_2PO_4$ | 0.5 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| $CaCl_2$ | 0.5 g |
| distilled water | 1 litre |

Potato Dextrose Broth (PDB) (Difco, Detroit, USA.)

| | |
|---|---|
| potato, infusion form | 200 g |
| bacto dextrose | 20 g |
| distilled water | 1 litre |

4.3 Reagents

- Sodium sulphate anhydrous ($NaSO_4$): Farmitalia Carlo Eaba
- Copper sulphate ($CuSO_4.5H_2O$): Farmitalia Carlo Eaba
- Magnesium sulphate ($MgSO_4.7H_2O$) Farmitalia Carlo Eaba
- Petroleum ether (b.p. 80-100°C) Mallinckordt Inc., St. Louis, USA
- Heptadecanoic acid ($C_{17}H_{33}COOH$): Sigma Chemical Co., St. Louis, USA
- Butylated hydroxytoluene (BHT): Sigma Chemical Co., St. Louis, USA
- Methanol: MERCK, Darmstadt, Germany
- HCl: MERCK, Darmstadt, Germany
- Heptadecanoic acid (C 17:0): Sigma Chemical Co., St. Louis, USA

4.4 Apparatus

- Gas chromatography analyses were performed using a FISONS instrument model HWD CONTROL, Model GC 8340-00. The conditions of operation were Helium carrier gas maintained at 70 psi, flow rate of carrier gas maintained at 55 $kg/cm^2$ column temperature 200°C, injector temperature 220°C, detector temperature

200 °C. Detector was sp. 2330 supelco fused silica capillary by length 60 m, diameter 0.25 mm. The thickness of surface was coat 0.2 µm. Type of injection was split mode.

- Low temperature incubator Model 815, Precision Scientific, USA

4.5 Procedure

4.5.1 Morphology on solid media at 22°C

The *Aschersonia* strains were maintained on PDA at room temperature (ca. 25°C). After they produced spores they were spread on fresh Petri dishes and incubated for one week. A 7 mm cork borer was used to transfer plugs into 55 mm Petri plates. Prepared, inoculated plates were then incubated at 22°C and observations of the morphology were made as well as growth characteristics on MEA, M 102, MSM, PDA and SDA.

4.5.2 Morphology in liquid media at 22°C

The method used for producing inoculum was the same as in the previous experiment. A 7 mm cork borer was used to transfer plugs into 250 ml Erlenmeyer flasks containing 10 ml of autoclaved liquid medium PDB, PYGM, SDB, MSM and SM at 22°C.

4.5.3 Effect of temperature on growth and sporulation on PDA

PDA was chosen to compare growth at six temperatures; 15, 20, 22, 25, 28 and 30°C. Temperature profiles were then determined.

4.5.4 The extraction of fatty acids.

The dry weight of biomass was determined by filtration through filter paper (Whatman qualitative circle diameter 125 mm, no.1) then washing with distilled water and drying at 80°C for 24 h. The lipids were extracted as follows:

Extraction procedure

Dried mycelia were powdered using a pestle and mortar

↓

Weight at least 100 mg in each sample

↓

Addition of 2 ml of methanolic [5%(v/v) HCl in methanol] and internal standard heptadecanoic (C17:0) 50 µl

↓

Heating at 80°C (about) 2 h.

↓

Addition of 1 ml BHT and 1 ml water and centrifuge

↓

Suck only oil at the top of the solution and

add sodium anhydrous sulphate to absorb water

Ready to inject into gas-chromatography

The use of gas-chromatography to characterize fatty acid profiles of lipids in biological material has been routinely done in laboratories of various scientific institutions and organizations. This process changes the volatility of lipid components and improves peak shape and thus provides better separation.

Many internal standards have been used in conjunction with the direct transmethylation method to quantify lipid components including tridecanoic acid (C13:0) [ 78 ] pentadecanoic acid (C15:0) [ 76 ], heptadecanoic acid (C17:0) [ 79 , 80 ] and nonadecanoic acid (C19:0) [ 81 , 82 ]. Heptadecanoic acid methyl ester has also been used when estimating an internal standard for a particular application. In selecting the standard one needs to obtain a balance between the following:

it represents the major fatty acids of interest in the lipids in terms of molecular size and structure, and

its chromatographic peak is not in the region where a number of peaks are in close proximity.

In addition the internal standard selected should be absent from the biological samples to be tested. When alkaline catalyst is used a free fatty acid cannot be used as an internal standard.

A triglyceride can substitute for the internal standard [ 83 ]. Regardless of which internal standard is to used it should be added at the beginning of the reaction. By doing so it is taken through all the steps of the procedure. Consequently, the need to account for completion of methylation and the dilution of the GC injection volume is eliminated.

Fatty acid composition was determined by identifying fatty acids of interest and comparing their relative retention times with the known standard. The fatty acids selected to be included in the normalised total area were those normally present in microbial oils and the analysis was only accepted as valid if the normalised area was greater than 90% of the total area under all peaks. The normalisation process is designed to eliminate background interference and noise from the results.

# Chapter 5

## Morphology and growth of *Aschersonia*:

## Results and discussion

### 5.1 Effect of media on growth of *Aschersonia*

The five strains of *Aschersonia* were grown at 22°C using the five media: MSM, MEA, PDA, SDA and M102. The results (Figs 5.1-5.5) show that all five strains grew well on PDA with M102 and SDA also producing good growth. Generally these fungi did not grow well on MSM.

Mitosporic fungi typically have low requirements and substantial growth of insect pathogenic *B. bassiana* and *M. anisopliae* can be obtained in media containing only dextrose, a nitrate and a macro-mineral solution [ 84 ]. However, semi-defined media (including protein hydrolases) or natural undefined substrates rich in starch (e.g. rice, oatmeal, potato) have proved to consistently give the highest yield [ 85 ]. In this study the five *Aschersonia* strains grew well on the undefined media PDA. Commonly, the composition of potato extract in 100 g/dw is starch, protein, fat, calcium, iron, vitamin B1, vitamin B2, vitamin C, nicotinic acid, thiamine, niacin, ascorbic acid, riboflavin and amino acids (lysine, methionine, cystine) [ 86 ].

*A. tubulata*-5877 did not show a clearly defined lag phase. For four of the five media their was growth from 3-20 days followed by a stationary phase (Fig. 5.1). MSM was different in that there was slow but steady growth over the duration of the experiment. From this it seems that on nutrient-poor media (such as MSM) the vegetative mycelium initially develops searching for nutrients. When the stationary phase was reached PDA had produced the most growth (25 mm) and MSM the least (10-15 mm). This suggests the vegetative hyphae on the nutrient-poor MSM became exhausted.

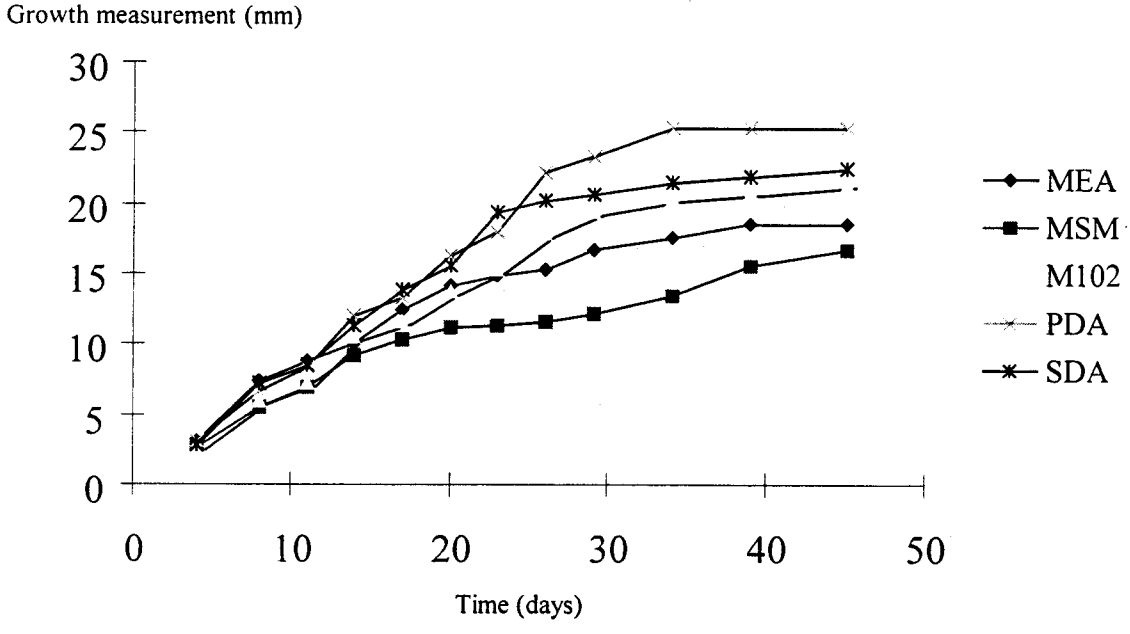


Fig. 5.1 Growth measurement on various media for *A. tubulata*-5877

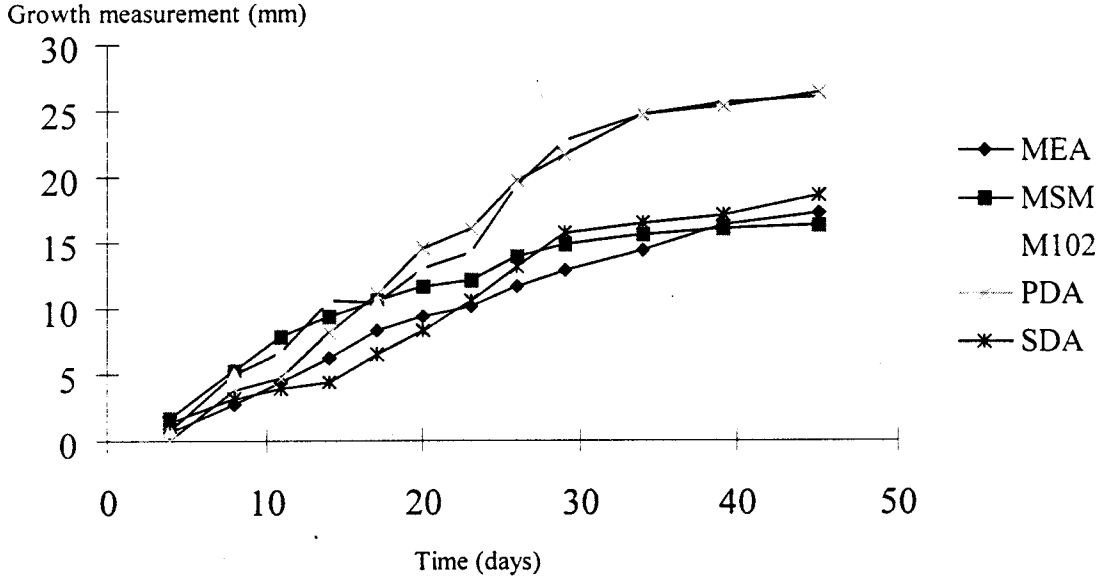


Fig. 5.2 Growth measurement on various media for *A. tubulata*-5996

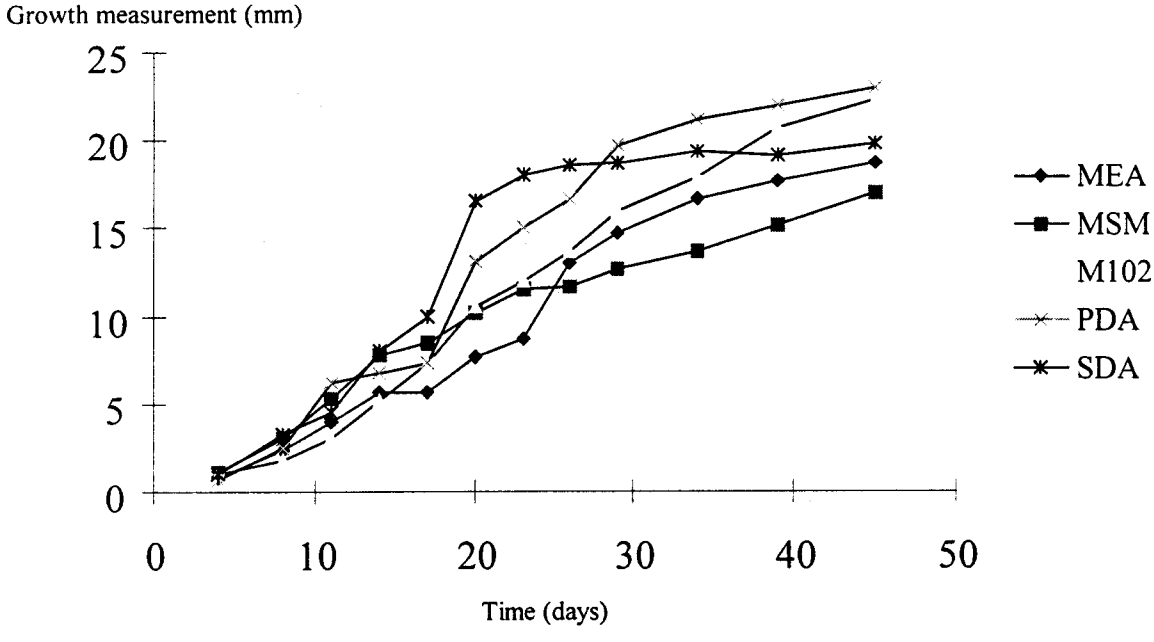


Fig. 5.3 Growth measurement on various media for *A. hypocreoidea*-3162

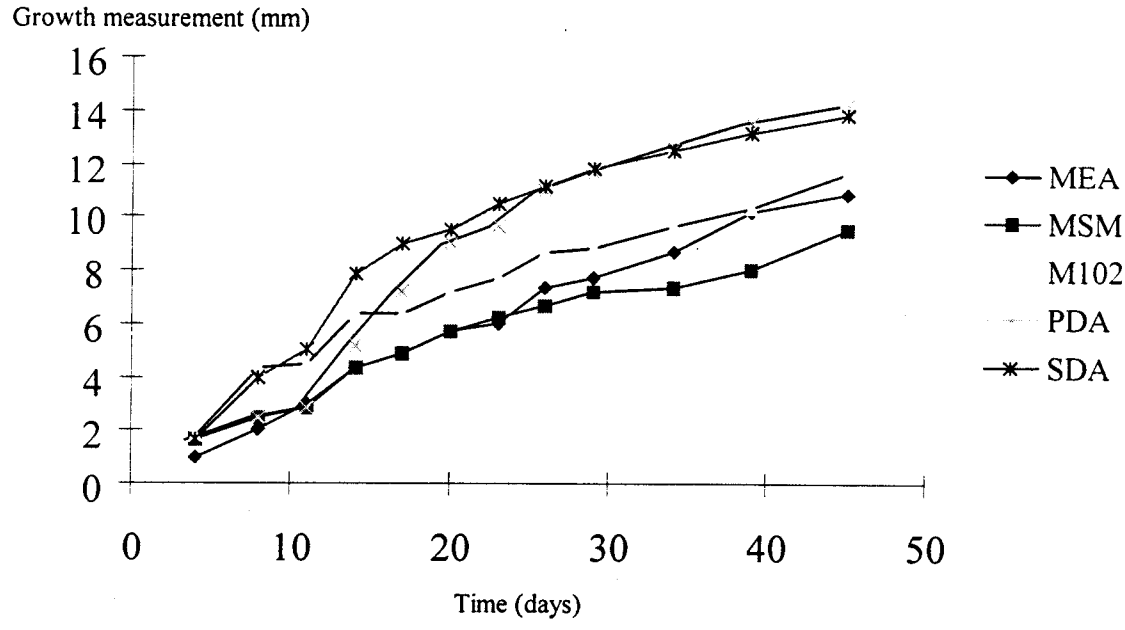


Fig. 5.4 Growth measurement on various media for *A. hypocreoidea*-5269

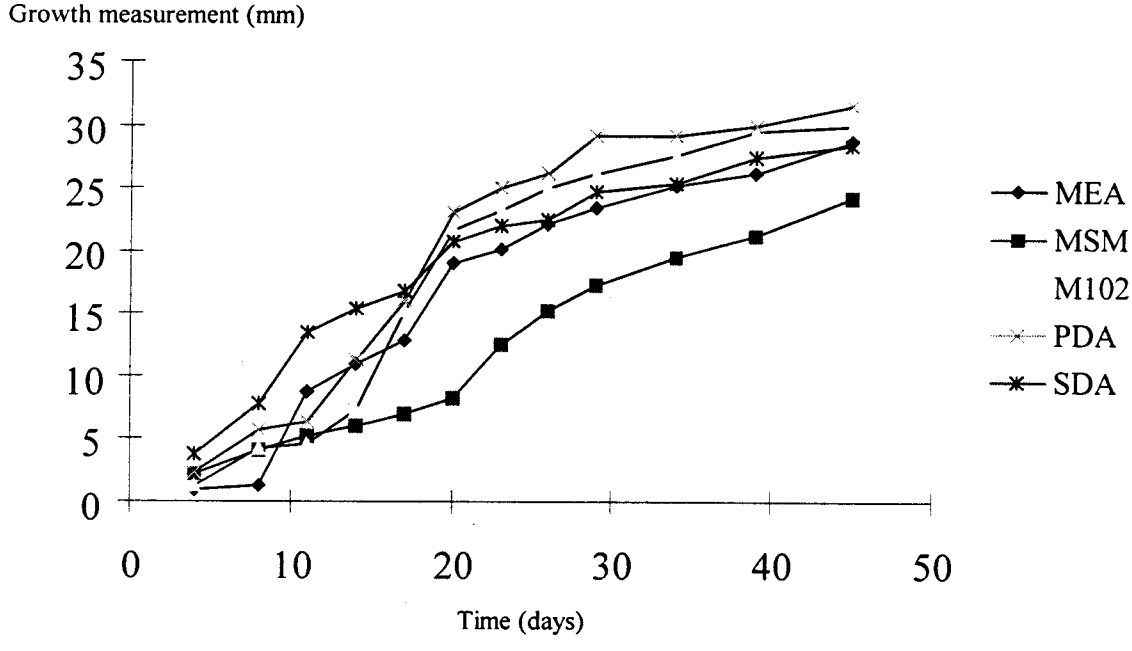


Fig. 5.5 Growth measurement on various media for *A. placenta*-5164

For *A. tubulata*-5996 (Fig. 5.2) this followed a similar pattern to *A. tubulata*-5877 (Fig. 5.1) with initially rapid development followed by a stationary phase at about 29 days. PDA and M102 had similar growth curves whereas MEA, SDA and MSM produced poorer growth (Fig. 5.2). In contrast to *A. tubulata* the two strains of *Aschersonia hypocreoidea* behaved differently (Fig 5.3 and Fig 5.4). *A. hypocreoidea*-3162 followed the same pattern as the two *Aschersonia tubulata* strains (Fig. 5.3) but *A. hypocreoidea*-5269 continued to grow on all five media over the course of the experiment. The two *A. tubulata* strains and *A. hypocreoidea*-3162 generally had colonies that were 15-25 mm on the five media after 45 days growth. However, *A. hypocreoidea*-5269 grew especially slowly and after 45 days colonies were only 8-13 mm diameter (Fig. 5.4) Again, for *A. hypocreoidea-A. hypocreoidea*-5269, PDA proved to be the best media with MSM the poorest. The single isolate of *Aschersonia placenta* showed a different response to the other two species (Fig. 5.5). Growth was generally better on all five media with colonies of 23-32 mm developing after 45 days. For the previous four strains it was not possible to clearly detect a lag phase which I assumed to be less than four days. However for *A. placenta*-5164 growing on MEA there was a clear lag phase of about 7 days (Fig. 5.5). Although MSM appeared the poorest media it is of note that slow growth seemed to continue after the 45 days of this experiment for all five strains.

5.2 Effect of media on sporulation.

The growth of all fungi including entomopathogenic species responds differently to nutrients in media. Especially it has been shown that the nutritional requirements of

entomopathogenic fungi can vary within species depending on the isolate [ 87 ]. If entomopathogenic fungi are to be used for biocontrol it is necessary to grow them on a media that will produce large numbers of spores. Following the effect of different media on the growth of the five strains I then looked at how these media affected sporulation.

In my work it is clear that media with minimal nutrients (e.g. MSM and SDA) are not good for spore production in the genus *Aschersonia*. Sporulation of the five strains occurred on PDA, M102 and MEA (Table 5.1). There was no sporulation on MSM for any of the five strains. It is of interest that on SDA that there was a variable sporulation for two species: *Aschersonia hypocreoidea* and *Aschersonia tubulata*. *A. hypocreoidea*-3162 sporulated on SDA while *A. hypocreoidea*-5269 did not. Similarly, *A. tubulata*-5877 sporulated on SDA but *A. tubulata*-5996 did not. Generally lower amounts of conidia were produced on MEA and M102 than on PDA (unpubl. obs.). PDA produced a great deal of conidia and one reason may be that the levels of carbon and nitrogen in the other media were not suitable for conidial production.

Latgé (1981) [ 87 ] noted that 'fungal growth is an autocatalytic process and the rate of growth depends on the individual organism and the physics and chemical conditions of the culture medium. These factors will influence both the mass transfer kinetics, especially at the gas-liquid interface and also the type of growth (yeast-like mycelial floc or pellet)'. A study of the effect of nitrogen and carbon sources on the sporulation of the entomopathogenic fungi in submerged culture has shown that the

yield of conidia depends on the composition of the medium. Sporulation of *Nomuraea rileyi* (Farlow) Samson is optimal in a Sabouraud Maltose Agar supplemented with 1% yeast extract [ 88 ]. A period of vegetative growth

invariably precedes sporulation [ 88 ]. The production of a large number of spores usually requires a well-nourished mycelium [ 43 ]. The nutrient concentration and quality that favours sporogenesis are often highly specific [ 43 ]. It is well-documented that conditions favouring spore formation are usually different and more restricted than those controlling mycelial growth [ 89 , 90 ]. This seems to be the case in this study where PDA gave both good growth and good sporulation but SDA gave good growth and variable sporulation.

Starvation or reduction in food supply usually stimulates sporulation in fungi and nitrogen is the first nutrient to be exhausted. This appears to be a defence against autolysis or the formation of non-viable spores [ 66 , 90 ]. However, sporulation can occur without any starvation of the mycelium. The production of conidia of the entomopathogenic fungi *Hirsutella thompsonii* Fischer and blastospores of *Verticillium lecanii* (Zimm.) Viégas in batch culture is parallel to that of mycelial growth [ 91 ]. Spores are widespread, ensuring that whenever a new substrate becomes available, fungi will always be there to colonize it. Fungi produce vegetative hyphae as long as there is sufficient nutrition and then concentrate on accumulating reserves of energy, some to be invested in producing more vegetative hyphae, some to be stored. Samson *et al.* (1988) [ 43 ] noted that the fungus switches into the reproductive mode and produces spores either:

- when food runs out,
- staling factors build up
- storage reserves reach an appropriate level, or
- specific environmental signals are received.

Some fungi, however, produce spores directly on the vegetative hyphae and this is the case for all five strains of the three *Aschersonia* species used in my study. Conidia of *Aschersonia* spp. germinated normally within 24 h on both Distilled Water Agar (DWA) and Potato Carrot Agar (PCA) usually with a single germ tube and formed slow-growing stromatic colonies reflecting many of the morphological characteristics of the *in vivo* fungus [ 92 ].

Table 5.1 Status of sporulation of *Aschersonia* strains on different solid media at 22°C.

| Strain | Media | Conidial production |
|---|---|---|
| *A. hypocreoidea*-3162 | PDA | + |
| | MSM | - |
| | M102 | + |
| | MEA | + |
| | SDA | + |
| *A. hypocreoidea*-5269 | PDA | + |
| | MSM | - |
| | M102 | + |
| | MEA | + |
| | SDA | - |
| *A. tubulata*-5877 | PDA | + |
| | MSM | - |
| | M102 | + |
| | MEA | + |
| | SDA | + |
| *A. tubulata*-5996 | PDA | + |
| | MSM | - |
| | M102 | + |
| | MEA | + |
| | SDA | - |
| *A. placenta*-5164 | PDA | + |
| | MSM | - |
| | M102 | + |
| | MEA | + |
| | SDA | + |

+ = sporulation , - = no sporulation

5.3 The effect of temperature on vegetative growth of *Aschersonia*

From the results of the effect of media on growth PDA was selected to compare the range of temperature suitable for growing the five strains of *Aschersonia*. In general the five strains grew best between 20 and 28°C (Figs 5.6-5.10) when they generally reached a plateau after about 29 days. Consequently 29 days was chosen to compare the optimal temperature for growth. The optimum incubation temperature which allowed good mycelial growth for all five strains of *Aschersonia* was between 25°C and 28°C. Although the genus *Aschersonia* is of tropical to sub-tropical distribution the five strains studied responded poorly to temperatures usually considered tropical. Especially, growth was poor at 30°C. Although day time temperatures in the forest are often above 30°C (Hywel-Jones, pers. comm.) night time temperatures are lower and this suggest that these fungi are physiologically active in the night time.

Temperature requirements may vary between species and also at the intraspecific level. For example strains of *Erynia neoaphidis* originating in the subtropical or tropical zones of Mexico and Brazil have an optimum of 26-28°C, whereas strains of the same species isolated in Northern Europe germinate best at 20-23°C [ 43 ]. For optimal development of most entomogenous fungi, however, temperature should be between 25 and 30°C [ 93 , 94 ]. In future it may be advisable to look at temperature profiles of *Aschersonia* strains taken from low elevation (where temperature is higher) and compare there with strains isolated from high elevation.

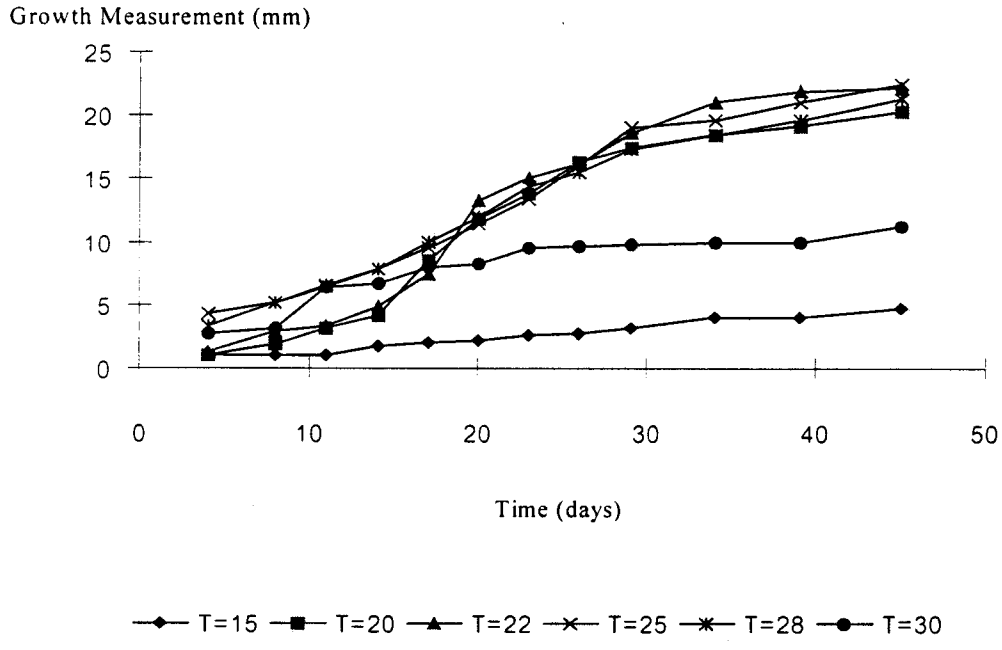


Fig. 5.6 Growth of *A. hypocreoidea*-3162 at various temperatures on PDA.

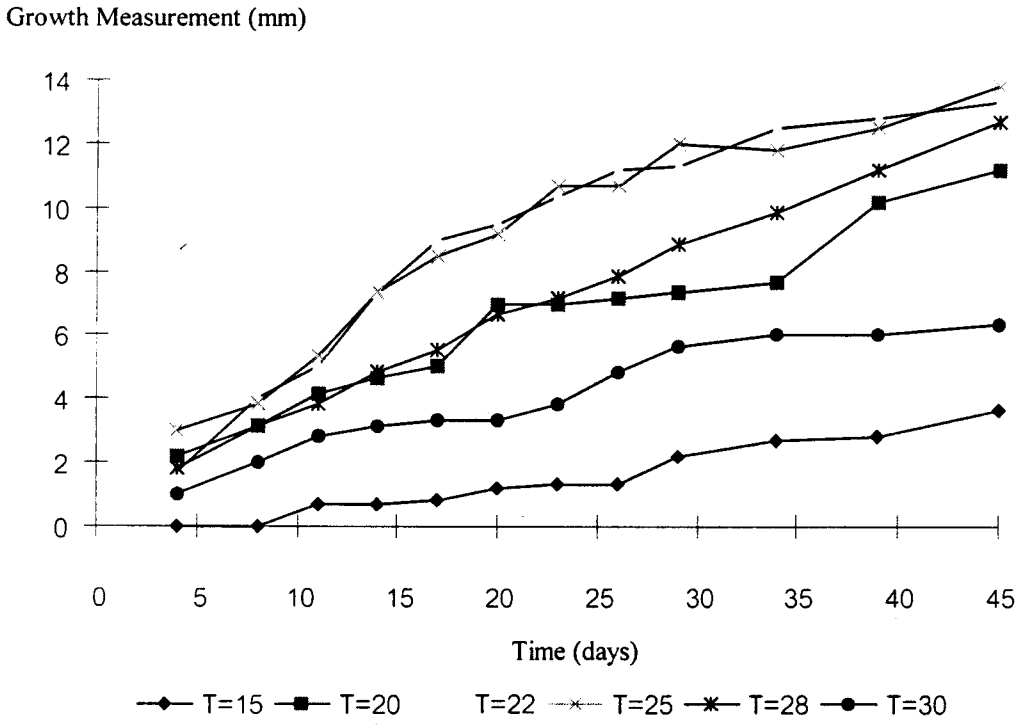


Fig. 5.7 Growth of *A. hypocreoidea*-5269 at various temperatures on PDA.

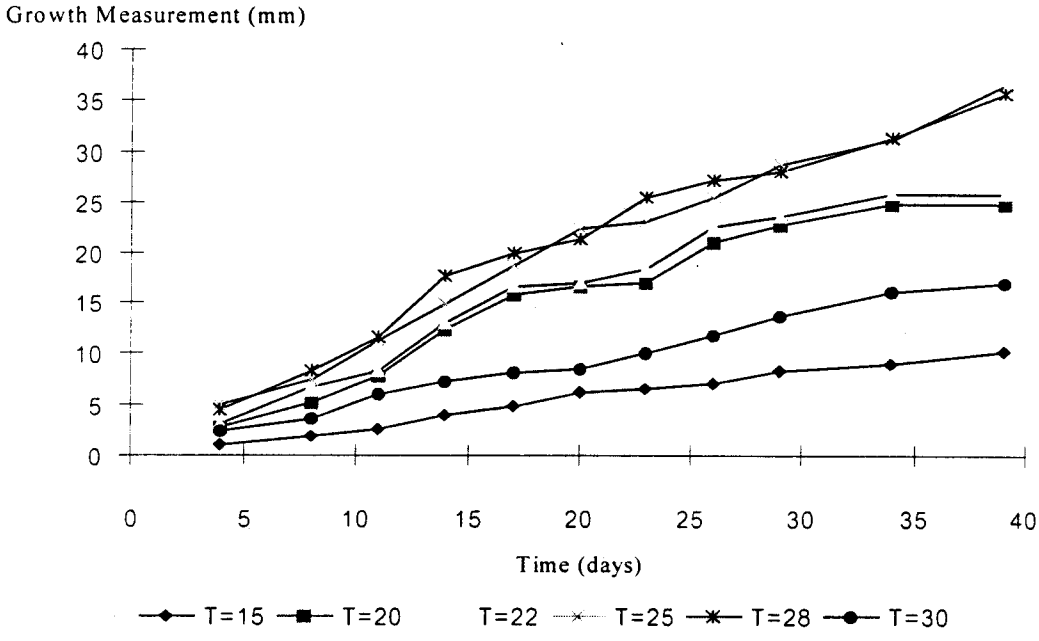


Fig. 5.8 Growth of *A. tubulata*-5877 at various temperatures on PDA.

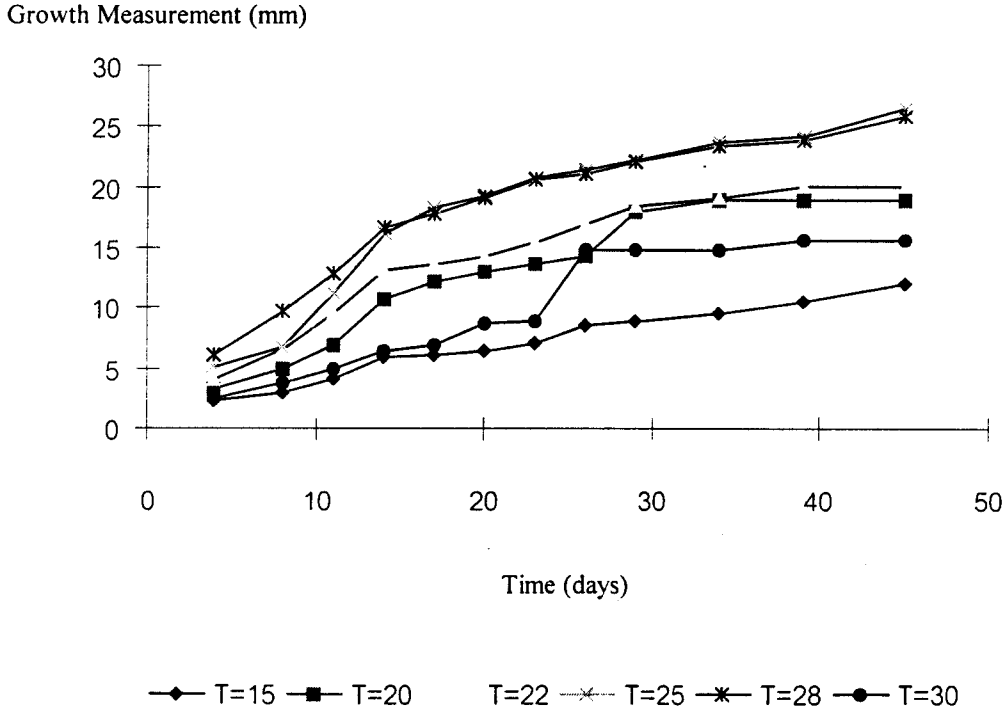


Fig. 5.9 Growth of *A. tubulata*-5996 at various temperatures on PDA.

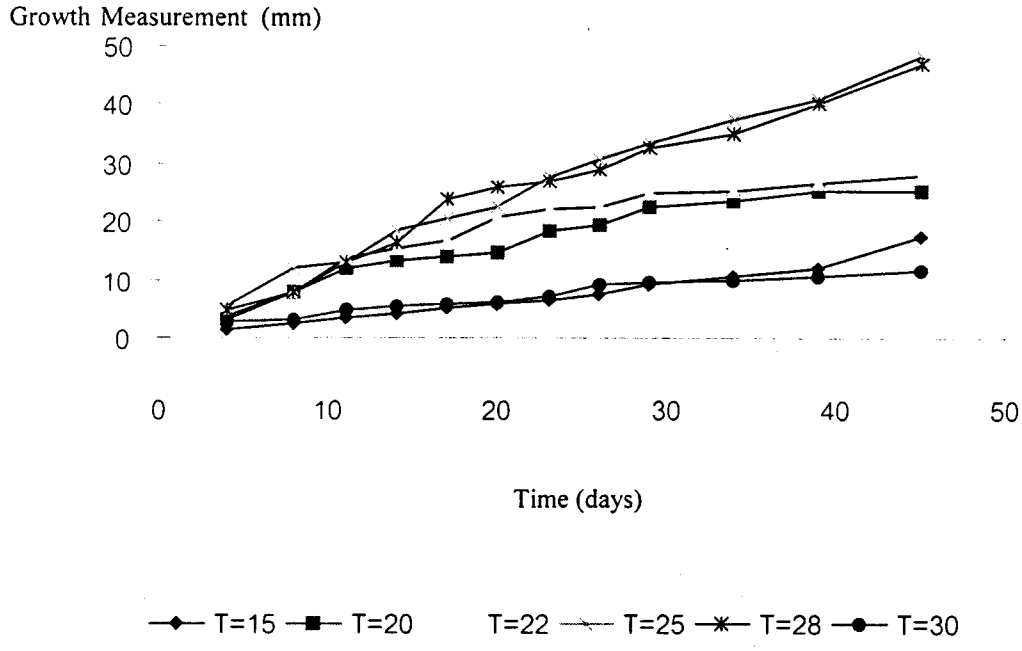


Fig. 5.10 Growth of *A. placenta*-5164 at various temperatures on PDA.

## 5.4 The effect of temperature on spore production of *Aschersonia*

As before PDA was the media chosen to examine the effect of temperature on spore production. On PDA the five strains showed a variable response and there appeared to be intraspecific variability at temperature extremes. All strains sporulated well at 20 and 22°C (Table 5.2). However at 15°C *A. hypocreoidea*-3162 did not sporulate whereas *A. hypocreoidea*-5269 did. Similarly, *A. tubulata*-5877 failed to sporulate at 15°C whereas *A. tubulata*-5996 did. It is of interest that the two isolates that could not sporulate at low temperature were isolated from forest that was close to sea level. Those that could sporulate at low temperatures were from forest of between 100 and 700 m elevation. *A. placenta*-5164 was an exception since this could sporulate at 15°C but was also isolated at sea level. Furthermore it can be noted that this strain

was able to sporulate over the whole temperature range selected for study. *A. placenta* is of widespread distribution and has been recorded from agricultural ecosystems such as mango and guava orchards in Thailand (Hywel-Jones, unpubl. obs., pers. comm.). It is possible that *A. placenta* is more widespread than other species of *Aschersonia* because of the wider range of temperatures at which it can sporulate. It is of note that at 25°C and 28°C the only isolate that could not sporulate was *A. hypocreoidea*-5269 which was isolated from Khao Yai at an elevation of 700 m. This isolate clearly preferred lower temperatures for sporulation. Above 28°C the ability to sporulate was restricted to two isolates (*A. tubulata*-5877 and *A. placenta*-5164) that came from Ko Charng at sea level where average temperatures are higher (Hywel-Jones, pers. comm.). The origin of the isolate therefore seems to affect sporulation response with isolates from sea level being able to sporulate at higher temperatures while isolates from higher elevations are able to sporulate at lower temperatures. Temperature optima for spore production can also be related to the role of the spore in the life cycle of the fungus. Although spores are the main target propagule in the production of myco-insecticides, the environmental factors governing the sporulation of the entomopathogenic fungi have been poorly studied. In particular, the optimum conditions for sporulation and for enhancing the pathogenicity or viability of the spores produced must be determined since it has been established that the environmental factors during fermentation influence both the aggressiveness of the spores and their survival [ 43 ]. When the humidity and temperature conditions are not optimal for sporulation, the fungus may however remain viable in the insect for several months. When conditions for supporting sporulation become present then the fungus can be re-activated [ 43 ].

Table 5.2 Effect of temperature on the sporulation of *Aschersonia*, on PDA.

| Temperature | Strain Number | | | | |
|---|---|---|---|---|---|
| | 3162 | 5269 | 5877 | 5996 | 5164 |
| 15 | - | + | - | + | + |
| 20 | + | + | + | + | + |
| 22 | + | + | + | + | + |
| 25 | + | - | + | + | + |
| 28 | + | - | + | + | + |
| 30 | - | - | + | - | + |

## 5.5 Morphology on solid media

### 5.5.1 Media effect on conidial size

In the past Petch (1921) [ 27 ] and others considered conidial size to be an important characteristic for separating species. This experiment studied the effect of each medium on conidial production and size of conidia (Table 5.3). It can be seen that nutrients clearly affect conidial size. Conidia were produced by all strains on PDA, MEA and M102 (Table 5.3). No conidia were produced by any strains on MSM and a variable response was noted for SDA. Conidia were produced on SDA by *A. hypocreoidea*-3162 but not by *A. hypocreoidea*-5269. Similarly, for *Aschersonia tubulata* conidia were produced by *A. tubulata*-5877 but not by *A. tubulata*-5996. Analysis of variance was used to test if there were significant differences between the mean lengths and widths of conidia produced by the strains on different media. For all five strains media had a highly significant effect on the length of conidia produced (Table 5.3 and Appendix D.). The media used had a slightly lesser effect on the conidial width. For *A. tubulata*-5877 and *A. placenta*-5164 there was a highly significant difference ($P<0.001$). In contrast for *A. tubulata*-5996 there was no significant difference ($P = 0.086$) in the conidial width. It is of note that PDA generally produced the smallest conidia whereas MEA produced the longest. The exception was *A. tubulata*-5996 where both PDA and MEA produced smaller conidia while M102 produced the longest.

The nutrient-rich media M102 and MEA both generally produced the largest conidia. Both of these were the only two media containing malt extract. A study of the effect

of nitrogen and carbon sources on the sporulation of entomopathogenic fungi in submerged culture has shown that the yield of conidia depends on the composition of the medium [ 95 ]. Species sharing subgeneric and generic features still differ from one another in many other distinct features. Genera producing elongate primary or secondary conidia are advantageous for the taxonomist in this respect, since they display three quantitative features-criteria of the species level:

a) conidia length

b) conidial diameter

c) conidial length/diameter ratio [ 96 ].

For *Aschersonia hypocreoidea*, Petch (1921) [ 27 ] reported the length of conidia to be 8-13 μm and the width 2-2.5 μm shape of conidia were narrow-oval, lanceolate. In the laboratory the two isolates used in this study had very different conidial lengths. For *A. hypocreoidea*-3162 conidia were 8.5-10.3 μm by 1.4-1.7 μm with a length/width ratio of 5.4-7.0. However, for *A. hypocreoidea*-5269 the length of conidia ranged from 14.0-16.6 μm by 2.1-2.2 μm, with a conidial length/width ratio of 6.6-7.4. For *Aschersonia tubulata*, Petch (1921) [ 27 ] recorded conidial length as 8-10 μm with a width of 1.5 μm. The shape of conidia was fusoid with ends blunt. In the laboratory the length of conidia of *A. tubulata*-5877 were 7.6-9.1 μm and the width was 1.1-1.7 μm with length/width ratio of 5.3-6.7 while *A. tubulata*-5996 had conidia of 8.9-9.9 μm by width 5.5-6.4 μm, with length/width ratio of 5.5-6.4. From my study the two isolates of *Aschersonia tubulata* fall well within the range reported

by Petch (1921). For *Aschersonia placenta* Petch (1921) [ 27 ] reported the length of conidia to be 10-14 μm by 1.5-2 μm and the shape of conidia to be fusoid with ends acute. In the laboratory for *A. placenta*-5164 conidia were 11.9-14.9 μm by 1.7-2.1 μ m with a length/width ratio of 6.7-7.4. The overall measurements again agree with those reported by Petch (1921) [ 27 ]. From these results the range in conidial size was generally agreeable with measurements reported by Petch (1921) [ 27 ]. An exception, however, was *A. hypocreoidea*-5269 which had longer conidia than Petch (1921) [ 27 ] had reported. While morphology is still important in support of taxonomy it is clear from my work that nutrients can affect conidial size. When specimens are collected in the field the researcher will have no knowledge of the nutrient status of the host insect. It is possible therefore that spore size could be affected by how well fed the host was before it was infected. For example in the dry season the scale insect might not get the same quality or quantity of nutrients from a leaf that it may get in the rainy season.

Table 5.3 The conidial size of five strains of *Aschersonia* on different solid media (for standard errors of the means as calculated from the ANOVA please refer to Appendix D.)

| Strain | Media | Length (μm) | width (μm) | Length/width ratio |
|---|---|---|---|---|
| *A. hypocreoidea*-3162 | M102 | 9.8 | 1.6 | 6.2 |
| | MEA | 10.3 | 1.5 | 7.0 |
| | PDA | 8.5 | 1.4 | 5.9 |
| | SDA | 9.0 | 1.7 | 5.4 |
| | | | | |
| *A. hypocreoidea*-5269* | M102 | 15.3 | 2.2 | 7.0 |
| | MEA | 16.6 | 2.2 | 7.4 |
| | PDA | 14.0 | 2.1 | 6.6 |
| | | | | |
| *A. tubulata*-5877 | M102 | 9.1 | 1.7 | 5.3 |
| | MEA | 9.1 | 1.5 | 6.0 |
| | PDA | 7.6 | 1.1 | 6.7 |
| | SDA | 9.1 | 1.6 | 5.6 |
| | | | | |
| *A. tubulata*-5996* | M102 | 9.9 | 1.6 | 6.4 |
| | MEA | 8.9 | 1.6 | 5.6 |
| | PDA | 9.0 | 1.6 | 5.5 |
| | | | | |
| *A. placenta*-5164 | M102 | 11.8 | 1.8 | 6.7 |
| | MEA | 14.9 | 2.1 | 7.0 |
| | PDA | 11.9 | 1.7 | 6.9 |
| | SDA | 12.8 | 1.7 | 7.4 |

*Note: There was no sporulation on SDA.

5.5.2 Temperature effect on conidial size

I next studied the effect of temperature on conidial size (Table 5.4). For this the five strains were again grown on PDA. For the five strains used incubation temperature had a highly significant effect ($p<0.001$) on the length of conidia produced (Table 5.4; appendix D). For three of the strains (*A. hypocreoidea*-3162, *A. tubulata*-5877 and *A. tubulata*-5996) there was also a highly significant difference ($p<0.001$) in the widths (Table 5.4; appendix D). The exceptions were *A. placenta*-5164 which had a significant difference at the $p<0.05$ level and *A. hypocreoidea*-5269 where there was no significant difference in conidial width at different temperatures (Table 5.4; appendix D). For each strain over the range of temperature where sporulation occurred longer conidia were generally produced at higher temperatures. The exception was at 30°C where conidia tended to become smaller again (Table 5.4: *A. tubulata*-5877 and *A. placenta*-5164). For *A. hypocreoidea*-3162 the length of conidia increased from 8.48 µm at 20°C to 9.85 µm at 28°C. This pattern was the same for all other strains. From this it is assumed that conidia produced in the hot season and on insects at sea level may be longer than conidia produced in the cool season and on insects living at a higher elevation.

5.6 Growth characteristics in liquid media

All the fungi were grown on PDA for seven days before inoculation. A 7mm plug was used to inoculate each of the five strains of *Aschersonia*. 10 ml of autoclaved liquid media of PDB, SDB, PYGM, MSM and SM were inoculated and incubated at

22°C in the dark for 14, 21 and 30 days. The results showed that all five strains of the three species grew on every media except on SM. There was a similar pattern to the growth characteristics of the five strains of *Aschersonia* as follows. Initially the mycelium scattered from the inoculation plug. Hyphae then grew out from this plug breaking into hyphal fragments. After a few days the liquid medium became turbid as the hyphal fragments developed. After 7 days hyphal fragments coalesced to form a broken, uniform mat. The submerged mycelium in the liquid medium was slimy and sticky. Aerial mycelium then appeared at the surface of the liquid medium forming a white velvety mycelium in strains *A. hypocreoidea*-3162, *A. tubulata*-5877 and *A. tubulata*-5996 or a white hairy mycelium for *A. placenta*-5164; and a yellow velvety mycelium for *A. hypocreoidea*-5269. The development over the first 7 days was rapid but after 14 days the growth was almost constant. Sporulation occurred from about 14 days. However, *A. hypocreoidea*-5269 behaved differently from the other four strains. Initially the mycelium scattered from the inoculation plug. Hyphae then grew out from this plug breaking into hyphal fragments. The pellet of mycelium grew individually and adhered to the bottom of the flask. After 11 days hyphal fragments coalesced to form a broken and non-uniform mat. The submerged mycelium in the liquid medium was slimy and sticky. The surface mat of aerial mycelium was covered with yellow velvety hyphae. The development after 11 days was fast until 17 days after which the growth was almost constant. Although there was no further growth of mycelium after 20 days sporulation did not occur until about 45 days.

Table 5.4 The conidial size of five strains of *Aschersonia* incubated at different temperatures (for standard errors of the means as calculated from the ANOVA please refer to Appendix D)

| Strain | Temperature (°C) | length (μm) | width (μm) | Length/width ratio |
|---|---|---|---|---|
| *A. hypocreoidea*-3162 | 20°C | 8.48 | 1.44 | 5.9 |
| | 22°C | 8.89 | 1.61 | 5.5 |
| | 25°C | 8.79 | 1.64 | 5.4 |
| | 28°C | 9.85 | 1.71 | 5.8 |
| | | | | |
| *A. hypocreoidea*-5269 | 15°C | 12.77 | 2.06 | 6.2 |
| | 20°C | 13.92 | 2.10 | 6.6 |
| | 22°C | 13.97 | 2.12 | 6.6 |
| | | | | |
| *A. tubulata*-5877 | 20°C | 7.59 | 1.13 | 6.7 |
| | 22°C | 8.96 | 1.64 | 5.5 |
| | 25°C | 9.28 | 1.57 | 5.9 |
| | 28°C | 9.49 | 1.29 | 7.4 |
| | 30°C | 8.25 | 1.74 | 4.7 |
| | | | | |
| *A. tubulata*-5996 | 15°C | 8.10 | 1.55 | 5.2 |
| | 20°C | 8.88 | 1.48 | 6.0 |
| | 22°C | 8.96 | 1.64 | 5.5 |
| | 25°C | 10.11 | 1.56 | 6.5 |
| | 28°C | 10.56 | 1.55 | 6.8 |
| | | | | |
| *A. placenta*-5164 | 15°C | 11.05 | 1.73 | 6.4 |
| | 20°C | 11.82 | 1.79 | 6.6 |
| | 22°C | 11.88 | 1.73 | 6.9 |
| | 25°C | 12.45 | 1.91 | 6.5 |
| | 28°C | 12.23 | 1.89 | 6.5 |
| | 30°C | 11.68 | 1.63 | 7.2 |

# Chapter 6

## Analysis of fatty acids

### 6. Raw Data

Raw data for comparison is presented in Appendix A and B.

### 6.1 Production of Fatty Acids by *Aschersonia*

All five strains of *Aschersonia* produced palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 (cis 9)) and linolenic acid (18:2 (cis 9,12)). The chromatograph was run for up to 50 minutes but no other peaks were detected suggesting that only these four fatty acids were produced in detectable amounts. These results agree with a study on fatty acids of another insect pathogen *Nomuraea rileyi* which produced the same fatty acids [ 97 ]. From the literature the cellular fatty acid composition of 100 different filamentous fungi, including oomycetes, zygomycetes, ascomycetes, basidiomycetes and sterile mycelia shows that the most common and abundant fatty acids extracted were palmitic, stearic, oleic and linolenic acids [ 98 ]. The surface of each species of insect larva differs both qualitatively and quantitatively as regards free fatty acids [ 99 ]. It is assumed that the growth environment of a larva will influence both the number and kind of fatty acid present on its cuticle [ 100 ]. More

recent work with the river-blindness vector *Simulium damnosum* (Diptera: Simuliidae) has demonstrated that this is the case [ 101 , 102 ].

6.2 The effect of media on fatty acids

The isolates were grown in 10 ml of PDB, PYGM, MSM and SDB and were incubated at 22°C. In this analysis variation of time (14, 21 and 30 days) was used as a covariate while strains (code) were nested within species to determine the effect of media. It is found that the five strains of the three species of *Aschersonia* generally produced less than 2% total fatty acid (Table 6.1a). Of the four media used in this study, PDB seemed to be the best, overall, producing 0.87-1.82% total fatty acids as percent dry weight cells (Table 6.1a) at 14, 21 and 30 days. In contrast, MSM seemed to be poor for total fatty acids producing 0.77-0.97% total fatty acids as percent dry weight cells. An exception was *A. tubulata*-5996 where MSM produced larger amounts of total fatty acids than either PYGM or SDB (Table 6.1a). Of the five strains, *A. tubulata*-5877 was the best at producing total fatty acids – maximum 1.82% as percent dry weight cells - whereas *A. hypocreoidea*-5269 produced a maximum of 0.94% total fatty acids as percent dry weight cells (Table 6.1a). It is of note, however, that *A. tubulata* was able to produce the largest amount of total fatty acids (1.82% for *A. tubulata*-5877 grown in PDB) and the smallest (0.42% for strain *A. tubulata*-5996 grown in SDB). For *A. tubulata* media appeared to have a significant effect on the production of total fatty acids (0.42-1.82%) whereas media seemed to have only a limited effect on fatty acid production for *A. hypocreoidea* (0.75-1.09% total fatty as percent dry weight cells). For the single strain of *A.*

*placenta* used in this study media seemed to have an intermediate effect on fatty acid production (Table 6.1a).

It seems that the length of incubation in the four media has, overall, little effect on the amount of total fatty acids produced by the five strains (Table 6.1b). *A. hypocreoidea*-3162, *A. tubulata*-5877 and *A. placenta*-5164 showed a slight decrease in total fatty acids from 14 to 30 days. In contrast, strains *A. hypocreoidea*-5269 and *A. tubulata*-5996 showed a slight increase in total fatty acids from 14 to 30 days (Table 6.1b). It is not possible to make any firm conclusion for species (Table 6.1c) because the two strains of *A. tubulata* and *A. hypocreoidea* both reacted differently to time (Table 6.1b).

Table 6.1a Effect of media on total fatty acid produced by various strains of *Aschersonia sp.* at different days.

| Strains | Media | Total fatty acids production as percent dry weight cells on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | PDB | 1.09 | 1.07 | 1.04 |
| | PYGM | 0.92 | 0.97 | 0.90 |
| | MSM | 0.77 | 0.79 | 0.77 |
| | SDB | 0.81 | 0.80 | 0.80 |
| | | | | |
| *A. hypocreoidea*-5269 | PDB | 0.87 | 0.91 | 0.94 |
| | PYGM | 0.79 | 0.82 | 0.85 |
| | MSM | 0.77 | 0.78 | 0.75 |
| | SDB | 0.88 | 0.91 | 0.91 |
| | | | | |
| *A. tubulata*-5877 | PDB | 1.82 | 1.75 | 1.50 |
| | PYGM | 1.21 | 1.04 | 0.97 |
| | MSM | 0.87 | 0.84 | 0.77 |
| | SDB | 1.73 | 1.63 | 1.52 |
| | | | | |
| *A. tubulata*-5996 | PDB | 1.36 | 1.45 | 1.26 |
| | PYGM | 0.52 | 0.48 | 0.43 |
| | MSM | 0.97 | 0.85 | 0.77 |
| | SDB | 0.42 | 0.76 | 0.58 |
| | | | | |
| *A. placenta*-5164 | PDB | 1.22 | 1.33 | 1.17 |
| | PYGM | 0.98 | 0.88 | 0.94 |
| | MSM | 0.97 | 0.89 | 0.93 |
| | SDB | 1.04 | 1.06 | 1.04 |

Table 6.1b Represents the strains means for total fatty acid of *Aschersonia sp.*, calculated from table 6.1a.

| Strains | Total fatty acids production as percent dry weight cells on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 0.9 | 0.91 | 0.88 |
| *A. hypocreoidea*-5269 | 0.83 | 0.86 | 0.86 |
| *A. tubulata*-5877 | 1.41 | 1.32 | 1.19 |
| *A. tubulata*-5996 | 0.82 | 0.89 | 0.90 |
| *A. placenta*-5164 | 1.05 | 1.04 | 1.02 |

Table 6.1c Represents the species means for total fatty acid of *Aschersonia sp.* calculated from table 6.1b.

| Species | Total fatty acids production as percent dry weight cells on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea* | 0.87 | 0.89 | 0.87 |
| *A. tubulata* | 1.12 | 1.11 | 1.05 |
| *A. placenta* | 1.05 | 1.04 | 1.02 |

6.2.1 The effect of media on the production of palmitic acid

In contrast to the total fatty acid production where the results showed that PDB produced the best overall levels for the five strains of *Aschersonia*, no single media produced consistently high levels of palmitic acid. For *A. hypocreoidea*-3162 and *A. tubulata*-5996 MSM was the best media for production of palmitic acid (Table 6.2a). However, for *A. hypocreoidea*-5269 and *A. placenta*-5164 PYGM was the best

media whereas for *A. tubulata*-5877 PDB was the best media. Significantly, where MSM was generally the worst media for total fatty acid production there was no single media that was poor for palmitic acid production (Table 6.2a). Where MSM was good for *A. hypocreoidea*-3162 and *A. tubulata*-5996 it was poor for *A. placenta*-5164. Also, where PYGM was good for *A. hypocreoidea*-5269 and *A. placenta*-5164 it was poor for *A. hypocreoidea*-3162 and *A. tubulata*-5996. Of the five strains used *A. tubulata*-5996 generally produced the largest amounts of palmitic acid (16.24-32.12% as percent of total fatty acids) (Table 6.2a). In contrast to *A. tubulata*-5996, *A. tubulata*-5877 was the poorest producer of palmitic acid (15.38-19.61% as percent of TFA) (Table 6.2a). The two strains of *Aschersonia hypocreoidea* (3162 and 5269) and the single strain of *Aschersonia placenta* (5164) produced similar levels of palmitic acid – generally in the range of 14.43-21.04% as percent of TFA (Table 6.2a).

It seems that the length of incubation time in the four media has, overall, little effect on the amount of palmitic acid produced by four of the five strains (Table 6.2b). For *A. hypocreoidea*-3162, *A. hypocreoidea*-5269, *A. tubulata*-5877 and *A. placenta*-5164 the level of palmitic acid from 14 to 30 days was in the range 17.26-20.54%. *A. tubulata*-5996, however, was an exception since the levels of palmitic acid fell noticeably from 26.77% as percent of TFA at 14 days down to 19.7% as percent of TFA at 30 days (Table 6.2b). In contrast to *A. tubulata*-5996 the other *A. tubulata* strain showed little response to time. The two *Aschersonia hypocreoidea* strains (3162 and 5269) showed a slight increase in palmitic acid from 14 to 30 days. As for *A. tubulata*, *A. placenta*-5164 showed little response to time (Table 6.2c). Overall for the species *Aschersonia hypocreoidea* seems to have increasing amounts

of palmitic acid over the incubation period. It is not possible to make any firm conclusion for *Aschersonia tubulata* since the two strains (5877 and 5996) reacted differently to time (Table 6.2c).

Table 6.2a Effect of media and time on palmitic acid produced by various strains of *Aschersonia sp.*

| Strain | Media | Palmitic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | PDB | 17.43 | 19.78 | 19.85 |
| | PYGM | 16.58 | 15.33 | 17.77 |
| | MSM | 24.66 | 25.77 | 23.23 |
| | SDB | 18.32 | 21.27 | 19.48 |
| | | | | |
| *A. hypocreoidea*-5269 | PDB | 15.43 | 16.09 | 15.85 |
| | PYGM | 20.36 | 19.32 | 21.12 |
| | MSM | 18.25 | 19.51 | 17.05 |
| | SDB | 17.34 | 18.78 | 19.28 |
| | | | | |
| *A. tubulata*-5877 | PDB | 24.85 | 26.11 | 22.74 |
| | PYGM | 18.32 | 19.29 | 19.61 |
| | MSM | 19.29 | 18.80 | 19.57 |
| | SDB | 17.41 | 15.38 | 17.39 |
| | | | | |
| *A. tubulata*-5996 | PDB | 18.38 | 19.70 | 17.55 |
| | PYGM | 16.24 | 18.48 | 17.16 |
| | MSM | 28.45 | 32.12 | 22.24 |
| | SDB | 24.08 | 21.76 | 21.84 |
| | | | | |
| *A. placenta*-5164 | PDB | 18.71 | 17.57 | 18.97 |
| | PYGM | 20.54 | 19.58 | 21.04 |
| | MSM | 15.57 | 14.43 | 14.58 |
| | SDB | 16.90 | 15.55 | 14.44 |

Table 6.2b Represents the strains means for palmitic acid of *Aschersonia sp.*, calculated from table 6.2a.

| Strains | Palmitic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 19.25 | 20.54 | 20.08 |
| *A. hypocreoidea*-5269 | 17.85 | 18.43 | 18.33 |
| *A. tubulata*-5877 | 19.97 | 19.90 | 19.83 |
| *A. tubulata*-5996 | 26.77 | 23.02 | 19.70 |
| *A. placenta*-5164 | 17.93 | 16.82 | 17.26 |

Table 6.2c Represents the species means for palmitic acid of *Aschersonia sp.*, calculated from table 6.2b.

| Species | Palmitic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea* | 18.55 | 19.49 | 20.33 |
| *A. tubulata* | 23.37 | 21.46 | 19.77 |
| *A. placenta* | 17.93 | 16.82 | 17.26 |

6.2.2 The effect of media on the production of stearic acid

The five strains of *Aschersonia* produced only small levels of stearic acid (3.38-10.51% as percent of TFA) (Table 6.3a). For four of the five strains (*A. hypocreoidea*-5269, *A. tubulata*-5877, *A. tubulata*-5996 and *A. placenta*-5164) MSM proved to be the best media for producing stearic acid whereas for *A. hypocreoidea*-3162 PYGM proved to be better 6.56-7.67% as percent of TFA versus 5.24-6.66% as percent of TFA) (Table 6.3a). In contrast, PDB was the worst media for *A.*

*hypocreoidea*-5269 and *A. tubulata*-5996 while SDB was the worst for *A. hypocreoidea*-3162 and *A. placenta*-5164. Where PYGM was good for *A. hypocreoidea*-3162 it was the poorest media for stearic acid production for *A. tubulata*-5877. It is of note that *A. tubulata*-5996 produced the largest amount of stearic acid (14 days/MSM/10.51%) and also the smallest level of stearic acid at (14 days/PDB/3.38%) (Table 6.3a). No other strains produced more than 10% stearic acid with only *A. placenta*-5164 reaching 9%. Media clearly appeared to have a significant effect on the stearic acid production of *A. tubulata*-5996 (3.38-10.51% as percent of TFA) while it had minimal effect on the other four strains (Table 6.3a).

It seems that the length of incubation time in the four media has, overall, little effect on the amount of stearic acid produced by the five strains (Table 6.3b). *A. hypocreoidea*-3162 and *A. hypocreoidea*-5269 showed no response to time while *A. tubulata*-5877 and *A. tubulata*-5996 showed a slight decrease in stearic acid from 14 to 30 days (Tables 6.3b & c). However, for *A. placenta*-5164 there seemed to be little change in stearic acid levels from 14-21 days but then a rise from 21 to 30 days (Tables 6.3b & c).

Table 6.3a Effect of media and time on stearic acid produced by various strains of *Aschersonia sp.*

| Strain | Media | Stearic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | PDB | 5.82 | 4.43 | 5.40 |
| | PYGM | 7.35 | 6.56 | 7.67 |
| | MSM | 5.76 | 6.66 | 5.24 |
| | SDB | 4.47 | 5.36 | 5.35 |
| | | | | |
| *A. hypocreoidea*-5269 | PDB | 5.22 | 5.82 | 6.10 |
| | PYGM | 7.71 | 8.69 | 7.49 |
| | MSM | 8.64 | 7.29 | 6.66 |
| | SDB | 6.55 | 6.94 | 7.58 |
| | | | | |
| *A. tubulata*-5877 | PDB | 6.25 | 5.74 | 7.05 |
| | PYGM | 3.20 | 4.18 | 4.07 |
| | MSM | 8.70 | 5.47 | 4.62 |
| | SDB | 7.22 | 6.68 | 3.73 |
| | | | | |
| *A. tubulata*-5996 | PDB | 5.35 | 3.38 | 3.73 |
| | PYGM | 6.14 | 4.35 | 5.30 |
| | MSM | 10.51 | 10.15 | 4.87 |
| | SDB | 7.65 | 9.08 | 7.50 |
| | | | | |
| *A. placenta*-5164 | PDB | 5.01 | 6.34 | 7.96 |
| | PYGM | 5.27 | 6.5 | 7.4 |
| | MSM | 7.54 | 6.76 | 6.64 |
| | SDB | 6.54 | 4.36 | 5.75 |

Table 6.3b Represents the strains means for stearic acid of *Aschersonia sp.*, calculated from table 6.3a.

| Strains | Stearic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 5.85 | 5.75 | 5.92 |
| *A. hypocreoidea*-5269 | 7.03 | 7.19 | 6.96 |
| *A. tubulata*-5877 | 6.34 | 5.32 | 4.87 |
| *A. tubulata*-5996 | 7.41 | 6.74 | 5.35 |
| *A. placenta*-5164 | 6.09 | 5.99 | 6.94 |

Table 6.3c Represents the species means for stearic acid of *Aschersonia sp.*, calculated from table 6.3b.

| Species | Stearic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea* | 6.44 | 6.47 | 6.44 |
| *A. tubulata* | 6.88 | 6.13 | 5.11 |
| *A. placenta* | 6.09 | 5.99 | 6.94 |

6.2.3 The effect of media on the production of oleic acid

These results showed that generally for four of the five strains oleic acid was produced at levels of 15-33% as percent of TFA (Table 6.4a). The exception was *Aschersonia tubulata*-5996 which produced both the lowest (6.27%) and the highest (34.51% as percent of TFA) levels of oleic acid as percent of TFA. SDB was the best media for producing oleic acid in three of the five strains (*A. hypocreoidea*-3162, *A.*

*tubulata*-5877 and *A. tubulata*-5996) although it was the worst for *A. placenta*-5164 (Table 11a). Generally PDB was the worst media for three of the five strains (*A. hypocreoidea*-3162, *A. tubulata*-5877 and *A. tubulata*-5996) although it was the best for *A. hypocreoidea*-5269 (Table 6.4a). The largest amount of oleic acid was produced by *A. tubulata*-5996 after 30 days in SDB (34.51%) (Table 6.4a). In contrast the same strain produced the smallest amount of oleic acid in PDB at 30 days (6.27%) (Table 6.4a). Media clearly affected the amount of oleic acid produced by *A. tubulata*-5996 while for the other four strains media had a lesser effect on the amount of oleic acid produced (Table 6.4a).

It seems that the length of incubation time in the four media had, overall, little effect on the amount of oleic acid produced by the five strains (Table 6.4b). This was especially so for strains of *A. hypocreoidea* and *A. tubulata* (Tables 6.4b & c). However, for *A. placenta* there was a slight increase in levels of oleic acid from 14-30 days (Tables 6.4b & c).

Table 6.4a. Effect of media and time on oleic acid produced by various strains of *Aschersonia sp.*

| Strain | Media | Oleic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | PDB | 16.05 | 16.97 | 15.40 |
| | PYGM | 16.85 | 17.37 | 18.31 |
| | MSM | 20.47 | 20.77 | 20.68 |
| | SDB | 25.07 | 22.17 | 24.03 |
| | | | | |
| *A. hypocreoidea*-5269 | PDB | 28.23 | 27.47 | 28.39 |
| | PYGM | 18.06 | 20.70 | 22.83 |
| | MSM | 20.72 | 22.48 | 23.42 |
| | SDB | 20.18 | 19.22 | 18.57 |
| | | | | |
| *A. tubulata*-5877 | PDB | 18.11 | 17.82 | 18.69 |
| | PYGM | 19.62 | 20.35 | 15.40 |
| | MSM | 27.22 | 25.33 | 23.36 |
| | SDB | 28.42 | 26.59 | 20.00 |
| | | | | |
| *A. tubulata*-5996 | PDB | 9.97 | 8.43 | 6.27 |
| | PYGM | 17.93 | 19.98 | 19.44 |
| | MSM | 22.07 | 16.89 | 16.61 |
| | SDB | 24.74 | 31.25 | 34.51 |
| | | | | |
| *A. placenta*-5164 | PDB | 27.03 | 25.69 | 28.37 |
| | PYGM | 24.49 | 25.64 | 26.70 |
| | MSM | 26.45 | 29.36 | 33.01 |
| | SDB | 22.96 | 24.67 | 26.55 |

Table 6.4b. Represents the strains means for stearic acid of *Aschersonia sp.*, calculated from table 6.4a.

| Strains | Oleic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 19.61 | 19.32 | 19.63 |
| *A. hypocreoidea*-5269 | 21.80 | 22.47 | 23.30 |
| *A. tubulata*-5877 | 23.34 | 22.50 | 19.36 |
| *A. tubulata*-5996 | 18.68 | 19.14 | 19.21 |
| *A. placenta*-5164 | 25.23 | 26.34 | 28.66 |

Table 6.4c. Represents the species means for stearic acid of *Aschersonia sp.*, calculated from table 6.4b.

| Species | Oleic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea* | 20.71 | 20.90 | 21.47 |
| *A. tubulata* | 21.01 | 20.82 | 19.29 |
| *A. placenta* | 25.23 | 26.34 | 28.66 |

6.2.4 The effect of media on the production of linoleic acid

In this study it is found that the five strains of the three species of *Aschersonia* produced the largest amount of linoleic acid compared with the other fatty acid components (36.15-71.99% as percent of TFA) (Table 6.5a). Of the four media there was no single media that was the best for production of linoleic acid (Table 6.5a). While PDB was the best media for production of linoleic acid for *A. hypocreoidea*-3162 and *A. tubulata*-5996 SDB was the best for *A.hypocreoidea*-5269 and *A.*

*placenta*-5164. PYGM, however, was the best for *A. tubulata*-5877. MSM proved to be poor for production of linoleic acid for strains *A. hypocreoidea*-3162, *A. tubulata*-5877 and *A. tubulata*-5996. Four of the five strains produced maximum levels of no more than ca. 60% as percent of TFA (*A. hypocreoidea*-3162 – 60.75%; *A. hypocreoidea*-5269 – 55.94%; *A. tubulata*-5877 – 60.58% and *A. placenta*-5164 – 55.4%). *A. tubulata*-5996 however performed differently. Three of the media are comparable with the results for the four other strains; maximum levels being 59.7% - PYGM, 56.25% - MSM and 55.4% - SDB as percent of TFA. In contrast, PDB produced levels of linoleic acid which were consistently above 65% with a maximum of 71.99% (Table 6.5a). In contrast the same strain – *A. tubulata*-5996 - produced the smallest amounts of linoleic acid when grown on SDB (30 days/36.15% as percent of TFA) (Table 6.5a). Media had a large effect on the amount of linoleic acid produced in *Aschersonia tubulata*-5996, *Aschersonia tubulata*-5877 and *Aschersonia hypocreoidea*-3162 while for *A. hypocreoidea*-5269 and 5164 media had a minimal effect on the production of linoleic acid (Table 6.5a).

It seems that the length of incubation time in the four media had, overall, little effect on the amount of linoleic acid produced by four of the five strains (Table 6.5b). However, for *A. tubulata*-5877 there was a slight increase in linoleic acid levels from 14 to 30 days. For the three species, incubation period had a minimal effect on *A. hypocreoidea* and *A. placenta* although there was a small increase in levels of linoleic acid for *A. tubulata* (Table 6.5c).

Table 6.5a. Effect of media and time on linoleic acid produced by various strains of *Aschersonia sp.*

| Strain | Media | Linoleic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | PDB | 60.36 | 58.33 | 59.36 |
| | PYGM | 59.23 | 60.75 | 56.25 |
| | MSM | 48.83 | 46.81 | 50.85 |
| | SDB | 52.15 | 51.21 | 51.26 |
| | | | | |
| *A. hypocreoidea*-5269 | PDB | 51.14 | 50.63 | 49.83 |
| | PYGM | 53.88 | 51.30 | 48.57 |
| | MSM | 52.40 | 50.73 | 52.88 |
| | SDB | 55.94 | 55.07 | 54.58 |
| | | | | |
| *A. tubulata*-5877 | PDB | 50.37 | 49.77 | 51.33 |
| | PYGM | 58.77 | 56.19 | 60.58 |
| | MSM | 44.80 | 50.561 | 52.47 |
| | SDB | 46.16 | 51.50 | 58.89 |
| | | | | |
| *A. tubulata*-5996 | PDB | 66.04 | 67.32 | 71.99 |
| | PYGM | 59.70 | 57.20 | 58.11 |
| | MSM | 38.98 | 40.85 | 56.25 |
| | SDB | 43.54 | 37.55 | 36.15 |
| | | | | |
| *A. placenta*-5164 | PDB | 49.26 | 50.41 | 44.86 |
| | PYGM | 49.71 | 48.29 | 44.96 |
| | MSM | 50.45 | 49.46 | 45.77 |
| | SDB | 53.61 | 55.40 | 53.27 |

Table 6.5b. Represents the strains means for linoleic acid of *Aschersonia sp.*, calculated from table 6.5a.

| Strains | Linoleic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 55.14 | 54.28 | 54.43 |
| *A. hypocreoidea*-5269 | 53.34 | 51.93 | 51.47 |
| *A. tubulata*-5877 | 50.03 | 51.99 | 55.87 |
| *A. tubulata*-5996 | 52.07 | 50.73 | 55.63 |
| *A. placenta*-5164 | 50.76 | 50.89 | 47.23 |

Table 6.5c. Represents the species means for linoleic acid of *Aschersonia sp.*, calculated from table 6.5b.

| Species | Linoleic acid production as percent of total fatty acids on | | |
|---|---|---|---|
| | 14 days | 21 days | 30 days |
| *A. hypocreoidea* | 54.24 | 53.11 | 52.95 |
| *A. tubulata* | 51.05 | 51.36 | 55.75 |
| *A. placenta* | 50.76 | 50.89 | 47.23 |

6.3 The effect of temperature on fatty acid composition

At the three temperatures chosen the five strains of *Aschersonia* generally produced less than 2% total fatty acids as percent dry weight cells (Table 6.6a). Of the three temperatures used in this study 22°C seemed to be the best, overall, producing 0.94-1.82% total fatty acids as percent dry weight cells at 14, 21 and 30 days (Table 6.6a). In contrast, 15°C was poor for total fatty acid production producing 0.77-1.11% total fatty acids as percent dry weight cells (Table 6.6a). Of the five strains, *A. tubulata*-

5877 was the best at producing total fatty acids as percent dry weight cells - maximum 1.82% total fatty acids as percent dry weight cells (22°C/14 dys) - whereas *A. hypocreoidea*-3162 produced a maximum of 1.07% total fatty acids as percent dry weight cells (22°C/21 dys) (Table 6.6a). For three of the five strains the maximum was produced after 21 days. These strains were *A. hypocreoidea*-3162, *A. tubulata*-5996 and *A. placenta*-5164. *A. hypocreoidea*-5269 produced the maximum level of total fatty acids as percent dry weight cells at 30 days whereas *A. tubulata*-5877 produced the maximum amount after 14 days. It seems that individual strains within a species perform differently with respect to production levels over time. As the temperature was increased from 15-22°C four of the five strains showed a general increase in the amount of total fatty acids produced (Table 6.6b). The exception was *A. hypocreoideai*-5269 where the maximum level of fatty acids was at 20°C. This level was almost double the amount produced at 15°C and about 1.5 times that of the level produced at 22°C. Interestingly, *A. hypocreoidea*-3162 showed a small response to temperature with an increase to the maximum of less than 1.28% as percent of dry weight cells total fatty acid as percent dry weight cells (Table 6.6b). For the overall species effect both *A. tubulata* and *A. placenta* showed a general rise in total fatty acids from 15-22°C (Table 6.6c). No firm conclusions could be made for *A. hypocreoidea* since the two isolates examined reacted differently.

Table 6.6a. Effect of temperature and time on total fatty acids produced by various strains of *Aschersonia sp.*

| Strain | Temperature (°C) | Total fatty acids production as percent dry weight cells on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 15 | 0.95 | 0.83 | 0.77 |
| | 20 | 0.86 | 0.77 | 0.86 |
| | 22 | 0.94 | 1.07 | 1.01 |
| | | | | |
| *A. hypocreoidea*-5269 | 15 | 0.86 | 0.91 | 0.94 |
| | 20 | 1.11 | 1.14 | 1.16 |
| | 22 | 1.23 | 1.25 | 1.33 |
| | | | | |
| *A. tubulata*-5877 | 15 | 0.88 | 0.91 | 0.97 |
| | 20 | 1.71 | 1.67 | 1.52 |
| | 22 | 1.82 | 1.76 | 1.64 |
| | | | | |
| *A. tubulata*-5996 | 15 | 1.11 | 0.97 | 0.88 |
| | 20 | 1.07 | 1.25 | 1.16 |
| | 22 | 1.29 | 1.41 | 1.30 |
| | | | | |
| *A. placenta*-5164 | 15 | 0.92 | 0.86 | 0.95 |
| | 20 | 1.18 | 1.03 | 1.18 |
| | 22 | 1.20 | 1.34 | 1.18 |

Table 6.6b. Represents the strains means for effect of temperature on total fatty acids of *Aschersonia sp.*, calculated from table 6.6a.

| Strains | Total fatty acids production as percent dry weight cells at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea*-3162 | 0.85 | 0.84 | 1.01 |
| *A. hypocreoidea*-5269 | 0.90 | 1.71 | 1.27 |
| *A. tubulata*-5877 | 0.92 | 1.63 | 1.74 |
| *A. tubulata*-5996 | 0.99 | 1.16 | 1.33 |
| *A. placenta*-5164 | 0.91 | 1.13 | 1.24 |

Table 6.6c. Represents the species means for effect of temperature on total fatty acids of *Aschersonia sp.*, calculated from table 6.6b.

| Species | Total fatty acids production as percent dry weight cells at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea* | 0.88 | 1.28 | 1.14 |
| *A. tubulata* | 0.96 | 1.40 | 1.54 |
| *A. placenta* | 0.91 | 1.13 | 1.24 |

6.3.1 The effect of temperature on production of palmitic acid

The lowest level of palmitic acid at 14.67% as percent of TFA is found at 20°C, whereas the comparable levels of palmitic acid as percent of TFA at 15.9% and at 15.29% are found at 15 and 22°C, respectively (Table 6.7a). *A. tubulata*-5996 was generally the best strain over the three temperatures tested producing a maximum of 26.4%. Interestingly, this level was reached after 14 days whereas the other four strains produced maximum levels at 21 or 30 days incubation (Table 6.7a). In contrast, *A. hypocreoidea*-5269 was the poorest strain at the three temperatures used producing a maximum level of 18.61% palmitic acid as percent of TFA at 15°C and 21 days incubation (Table 6.7a). For the two *A. hypocreoidea* strains temperature had little effect on levels of palmitic acid produced (Table 6.7b). However, for the two *A. tubulata* strains response differed with one (5877) showing an increase in levels of palmitic acid from 15-22°C and the other - 5996 - showing a decrease. The one strain of *A. placenta* used in the study was similar to *A. tubulata*-5877 in having increasing levels of palmitic acid from 15-22°C (Table 6.7b). For the overall species effect *A. tubulata* and *A. placenta* showed similar responses with increasing temperature leading to increased levels of palmitic acid. *A. hypocreoidea* was different in that the level of palmitic acid decreased from 15-22°C (Table 6.7c) This result showed that temperature affected palmitic acid production only slightly for *A. hypocreoidea*-3162, *A. hypocreoidea*-5269 and *A. placenta*-5164 (Table 6.7a). For each of these the range was less than 4%. For *A. tubulata*-5877 when the temperature was higher it seemed to produce more palmitic acid after 14 and 21 days incubation although at 30 days 22°C there was a decrease in the amount of palmitic acid produced (Table 6.7a).

In contrast *A. tubulata*-5996 produced less palmitic acid when the temperature was higher (Table 6.7a). *A. tubulata*-5996 produced the largest amount of palmitic acid after 14 days at 15°C (Table 6.7a) whereas *A. hypocreoidea*-5269 produced the lowest level of palmitic acid after 14 days and at 20°C.

It seemed that over the range of temperature studied palmitic acid levels as percent of TFA varied slightly within strains (Table 6.7b). However, there was a large difference between strains with *A. hypocreoidea*-5269 having a minimum of 15.27% at 20°C compared with *A. tubulata*-5877 which had a maximum of 24.12% at 22°C. *A. hypocreoidea*-3162, *A. hypocreoidea*-5269 and *A. placenta*-5164 all had palmitic acid levels that varied by less than 2% from 15-22°C. However, the two *A. tubulata* strains had palmitic acid levels that varied by more than 3%. For the species overall temperature affected generally a less than 2% change in palmitic acid levels (Table 6.7c). For *A. tubulata* there was overall a 0.9% difference from 15-22°C, this due to an increase of palmitic acid level from 15-22°C in *A. tubulata*-5877 (Table 6.7b), whereas, *Aschersonia tubulata*-5996 showed a decrease in palmitic acid level from 15-22°C (Table 6.7b)

### 6.3.2 The effect of temperature on production of stearic acid

Stearic acid was produced in the smallest amounts by the five strains of *Aschersonia* used in this study (3.56-8.49% as percent of TFA). Temperature and length of incubation time generally had little effect on the amount of stearic acid produced by the strains of *Aschersonia* (Table 6.8a). *A. tubulata*-5877 produced the largest

amount of stearic acid as percent of TFA (8.49% after 21 dys at 20°C) whereas *A. placenta*-5164 produced the smallest amount of stearic acid as percent of TFA (3.56% after 14 dys at 15°C) (Table 6.8a). Four of the five strains had a range of 3-4% although *A. hypocreoidea*-5269 had a range of only 1%. For four of the five strains temperature had little overall effect on the level of stearic acid with this being 2% or less (Table 6.8b). However, for *A. tubulata*-5996 the range in stearic acid was 2.7% from 15-22°C. Interestingly, the other *A. tubulata* strain (*A. tubulata*-5877) had the smallest range (0.9%) (Table 6.8b). For *A. hypocreoidea*, overall, there seemed to be little difference in the amount of stearic acid produced from 15-22°C with a range of 1.2% (Table 6.8c). For *A. tubulata* there seemed to be a general decrease from 15-22°C but again with a small range of only 1.5%. In contrast, *A. placenta* had a range of 2% and showed a general increase in the level of stearic acid (Table 6.8c).

Table 6.7a. Effect of temperature and time on palmitic acid produced by various strains of *Aschersonia sp.*

| Strain | Temperature (°C) | Palmitic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 15 | 19.16 | 20.24 | 19.35 |
| | 20 | 19.66 | 20.67 | 22.13 |
| | 22 | 18.80 | 19.09 | 20.01 |
| | | | | |
| *A. hypocreoidea*-5269 | 15 | 17.62 | 18.61 | 16.26 |
| | 20 | 14.67 | 15.51 | 15.62 |
| | 22 | 15.29 | 15.78 | 15.99 |
| | | | | |
| *A. tubulata*-5877 | 15 | 18.95 | 16.72 | 22.04 |
| | 20 | 21.81 | 23.57 | 23.04 |
| | 22 | 24.80 | 26.18 | 21.39 |
| | | | | |
| *A. tubulata*-5996 | 15 | 26.40 | 21.49 | 18.45 |
| | 20 | 19.71 | 22.21 | 19.39 |
| | 22 | 19.28 | 19.43 | 18.43 |
| | | | | |
| *A. placenta*-5164 | 15 | 16.09 | 15.90 | 17.77 |
| | 20 | 16.56 | 17.73 | 16.28 |
| | 22 | 18.66 | 18.06 | 18.63 |

Table 6.7b. Represents the strains means for palmitic acid of *Aschersonia sp.*, calculated from table 6.7a.

| Strains | Palmitic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea*-3162 | 19.58 | 20.82 | 19.30 |
| *A. hypocreoidea*-5269 | 17.50 | 15.27 | 15.69 |
| *A. tubulata*-5877 | 19.24 | 22.81 | 24.12 |
| *A. tubulata*-5996 | 22.11 | 20.44 | 19.05 |
| *A. placenta*-5164 | 16.59 | 16.86 | 18.45 |

Table 6.7c. Represents the species means for palmitic acid of *Aschersonia sp.*, calculated from table 6.7b.

| Species | Palmitic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea* | 18.54 | 18.05 | 17.50 |
| *A. tubulata* | 20.68 | 21.63 | 21.59 |
| *A. placenta* | 16.59 | 16.86 | 18.45 |

6.3.3 The effect of temperature on production of oleic acid

Oleic acid was affected by time and temperature as the range varied from 7.8-31.29% (Table 6.9a). Strains *A. hypocreoidea*-3162 and *A. placenta*-5164 were similar in that they never produced oleic acid less than 20% as percent of TFA (minimum 22.02% for *A. placenta*-5164 after 30 days at 15°C). In contrast, for the other three strains these all produced levels of oleic acid as percent of TFA that were less than 20% except for *A. hypocreoidea*-5269 where 20% was barely produced after 30 days at

20°C. *A. tubulata*-5996 produced the lowest level of oleic acid as percent of TFA (7.8% after 30 days at 22°C) whereas *A. placenta*-5164 produced the highest level (31.29% after 30 days at 20°C) (Table 6.9).

Table 6.8a. Effect of temperature and time on stearic acid produced by various strains of *Aschersonia sp.*

| Strain | Temperature (°C) | Stearic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 15 | 5.39 | 6.53 | 7.44 |
| | 20 | 6.52 | 7.38 | 7.23 |
| | 22 | 5.20 | 4.55 | 5.43 |
| | | | | |
| *A. hypocreoidea*-5269 | 15 | 6.52 | 6.54 | 6.13 |
| | 20 | 6.24 | 5.51 | 6.11 |
| | 22 | 5.44 | 5.64 | 5.53 |
| | | | | |
| *A. tubulata*-5877 | 15 | 6.05 | 6.30 | 7.56 |
| | 20 | 5.89 | 8.49 | 7.72 |
| | 22 | 6.65 | 5.97 | 6.77 |
| | | | | |
| *A. tubulata*-5996 | 15 | 6.14 | 8.01 | 7.73 |
| | 20 | 6.35 | 6.39 | 5.82 |
| | 22 | 4.96 | 4.24 | 4.49 |
| | | | | |
| *A. placenta*-5164 | 15 | 3.56 | 4.55 | 4.91 |
| | 20 | 5.45 | 4.56 | 5.56 |
| | 22 | 4.90 | 6.61 | 7.51 |

Table 6.8b. Represents the strains means for stearic acid of *Aschersonia sp.*, calculated from table 6.8a.

| Strains | Stearic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea*-3162 | 6.45 | 7.04 | 5.06 |
| *A. hypocreoidea*-5269 | 6.40 | 5.95 | 5.54 |
| *A. tubulata*-5877 | 6.64 | 7.37 | 6.46 |
| *A. tubulata*-5996 | 7.29 | 6.19 | 4.56 |
| *A. placenta*-5164 | 4.34 | 5.19 | 6.34 |

Table 6.8c. Represents the species means for stearic acid of *Aschersonia sp.*, calculated from table 6.8b.

| Species | Stearic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea* | 6.43 | 6.50 | 5.30 |
| *A. tubulata* | 6.97 | 6.78 | 5.51 |
| *A. placenta* | 4.34 | 5.19 | 6.34 |

*A. hypocreoidea*-3162, *A. hypocreoidea*-5269 and *A. tubulata*-5877 were all similar in having an overall range of less than 4%. Furthermore, it seemed that these three strains were little affected by time or temperature. In contrast, *A. tubulata*-5996 and *A. placenta*-5164 had overall ranges of 9-10% suggesting that temperature and time affected the oleic acid levels of these significantly. For *A. tubulata*-5996 at 14 and 21 days there was little difference in oleic acid levels for 15 and 20°C. However, at 14 and 21 days there was a drop in oleic acid levels as percent of TFA to less than 10% (Table 6.9a). The response at 30 days was however different with a steep rise from

9.57% to 17.59% from 15-20°C followed by a drop to 7.8% at 22°C. For *A. placenta*-5164 there was generally little temperature effect at 14 and 21 days. However, at 30 days the oleic acid level as percent of TFA changed from the smallest amount for this strain (22.02%) at 15°C to the largest level (31.29%) at 20° C before again dipping to 28.62% at 22°C.

It seemed that temperature had little effect on the amount of oleic acid produced by *A. hypocreoidea*-3162, *A. hypocreoidea*-5269 and *A. tubulata*-5877 with all three having ranges of less than 1.6% (Table 6.9b). In contrast, *A. tubulata*-5996 and *A. placenta*-5164 both had ranges more than 5%. For *A. tubulata*-5996 there was a slight increase in oleic acid levels as percent of TFA from 15-20°C (12.17-14.88%) followed by a sharp decrease at 22°C (to 8.79%) (Table 6.9b). For *A. placenta*-5164 there was similarly a rise from 15-20°C (23.05-28.16%) with the oleic acid level dipping only slightly at 22°C (27.76%) (Table 6.9b). For the species overall *A. hypocreoidea* showed only a very slight effect with temperature (22.24-23.54%) while *A. tubulata* and *A. placenta* both had greater ranges (3.6 and 5.1% respectively) (Table 6.9c).

Table 6.9a. Effect of temperature and time on oleic acid produced by various strains of *Aschersonia sp.*

| Strain | Temperature (°C) | Oleic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 15 | 26.58 | 26.95 | 26.26 |
| | 20 | 27.52 | 28.70 | 27.99 |
| | 22 | 28.56 | 27.96 | 28.20 |
| | | | | |
| *A. hypocreoidea*-5269 | 15 | 17.41 | 18.01 | 18.21 |
| | 20 | 18.75 | 17.65 | 20.55 |
| | 22 | 18.24 | 17.32 | 16.59 |
| | | | | |
| *A. tubulata*-5877 | 15 | 18.43 | 19.02 | 19.08 |
| | 20 | 19.02 | 19.43 | 19.49 |
| | 22 | 18.33 | 17.93 | 18.62 |
| | | | | |
| *A. tubulata*-5996 | 15 | 15.06 | 11.88 | 9.57 |
| | 20 | 15.46 | 11.62 | 17.59 |
| | 22 | 9.57 | 8.99 | 7.80 |
| | | | | |
| *A. placenta*-5164 | 15 | 23.41 | 23.72 | 22.02 |
| | 20 | 25.54 | 27.65 | 31.29 |
| | 22 | 26.92 | 27.75 | 28.62 |

Table 6.9b. Represents the strains means for oleic acid of *Aschersonia sp.*, calculated from table 6.9a.

| Strains | Oleic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea*-3162 | 26.60 | 28.07 | 28.24 |
| *A. hypocreoidea*-5269 | 17.88 | 18.98 | 17.38 |
| *A. tubulata*-5877 | 18.84 | 19.31 | 18.29 |
| *A. tubulata*-5996 | 12.17 | 14.88 | 8.79 |
| *A. placenta*-5164 | 23.05 | 28.16 | 27.76 |

Table 6.9c. Represents the species means for oleic acid of *Aschersonia sp.*, calculated from table 6.9b.

| Species | Oleic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea* | 22.24 | 23.53 | 22.81 |
| *A. tubulata* | 15.51 | 17.10 | 13.54 |
| *A. placenta* | 23.05 | 28.16 | 27.76 |

6.3.4 The effect of temperature on production of linoleic acid

Of the four fatty acids linoleic acid was produced in the largest amounts as percent of TFA (45.4-69.39%). For the five strains *A. tubulata*-5996 was most affected by temperature and time with a range from 52.41-69.39% (Table 6.10a). In contrast, for *A. hypocreoidea*-3162, *A. tubulata*-5877 and *A. placenta*-5164 the range was about 10% and for *A. hypocreoidea*-5269 it was less than 4% (47.91-51.59%). Whereas *A. tubulata*-5996 produced linoleic acid as percent of TFA a maximum of 69.39% *A. hypocreoidea*-5269 was the poorest producer with a maximum of 51.59% (Table 6.10a). The worst producer of linoleic acid overall, however, was *A. placenta*-5164 with a low level of 45.4% (Table 6.10a). It is of note that at 22°C *A. placenta*-5164 consistently produced less than 50% linoleic acid whereas *A. tubulata*-5996 consistently produced more than 65% as percent of TFA.

It seems that temperature had little effect on *A. hypocreoidea*-5269 whereas there was an effect for the other four strains (Table 6.10b). *A. hypocreoidea*-3162 and *A. tubulata*-5877 had a similar response in that the levels of linoleic acid dipped from 15-20°C before rising again at 22°C. Interestingly *A. tubulata*-5996 and *A. placenta*-5164 showed different patterns with the former showing a sharp rise at 22°C while the latter showed a sharp decrease from 15°C to 20°C with a further, smaller decrease from 20-22°C (Table 6.10b). It was not possible to make any firm conclusions with regard to species since the two *A. hypocreoidea* and two *A. tubulata* strains both behaved differently (Table 6.10b&c).

Table 6.10a. Effect of temperature and time on linoleic acid produced by various strains of *Aschersonia sp.*

| Strain | Temperature (°C) | Linoleic acid production as percent of total fatty acids on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| *A. hypocreoidea*-3162 | 15 | 58.05 | 55.24 | 55.01 |
| | 20 | 55.08 | 54.31 | 49.42 |
| | 22 | 57.36 | 59.04 | 58.08 |
| | | | | |
| *A. hypocreoidea*-5269 | 15 | 49.28 | 47.91 | 51.36 |
| | 20 | 51.59 | 50.29 | 50.29 |
| | 22 | 50.72 | 50.63 | 50.29 |
| | | | | |
| *A. tubulata*-5877 | 15 | 56.58 | 57.99 | 53.31 |
| | 20 | 53.29 | 48.52 | 49.76 |
| | 22 | 50.24 | 49.93 | 53.23 |
| | | | | |
| *A. tubulata*-5996 | 15 | 52.41 | 58.63 | 64.25 |
| | 20 | 58.49 | 59.79 | 57.21 |
| | 22 | 66.20 | 67.34 | 69.39 |
| | | | | |
| *A. placenta*-5164 | 15 | 56.95 | 55.84 | 55.31 |
| | 20 | 52.46 | 50.06 | 46.93 |
| | 22 | 49.53 | 47.59 | 45.40 |

Table 6.10b. Represents the strain means for linoleic acid of *Aschersonia sp.*, calculated from table 6.10a.

| Strains | Linoleic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea*-3162 | 56.10 | 52.94 | 58.16 |
| *A. hypocreoidea*-5269 | 49.52 | 50.72 | 50.55 |
| *A. tubulata*-5877 | 55.96 | 50.52 | 51.13 |
| *A. tubulata*-5996 | 58.43 | 58.50 | 67.64 |
| *A. placenta*-5164 | 56.03 | 49.82 | 47.51 |

Table 6.10c. Represents the species means for linoleic acid of *Aschersonia sp.*, calculated from table 6.10b.

| Species | Linoleic acid production as percent of total fatty acids at | | |
|---|---|---|---|
| | 15°C | 20°C | 22°C |
| *A. hypocreoidea* | 52.81 | 51.83 | 54.36 |
| *A. tubulata* | 57.20 | 54.51 | 59.39 |
| *A. placenta* | 56.03 | 49.82 | 47.51 |

## 6.4 Discussion

### 6.4.1. Fatty acid production in the genus *Aschersonia*

The only other work where fatty acids have been examined in insect fungi was by Tyrrell [ 21 , 103 , 104 ]. The results from the study on *Aschersonia* differ clearly from those of Tyrrell. Tyrrell recorded a larger range of fatty acids (10 or 12% for individual species) as well as shorter-chained fatty acids which is in common with

studies on other members of the so-called lower fungi. Stahl & Klug (1996) [ 98 ] studied a range of fungi from the major taxonomic groups and recorded almost twenty fatty acids with chain lengths from 12 to 24 carbon atoms. Of 28 named species included in their study, only two species had profiles limited to the four fatty acids observed in this study. These two species were *Acremonium persicinum* and *Aspergillus alliaceus*. Although these two species are not insect pathogens (Hywel-Jones, pers. comm.) they are anamorphs of Clavicipitales and Eurotiales respectively. These two orders are closely related and with the related Hypocreales are the subject of study at BIOTEC (Hywel-Jones, unpubl. obs.). It is interesting that from the study by Stahl & Klug (1996) [ 98 ] three *Penicillium* species and a *Paecilomyces* species had a greater diversity of fatty acids (6-10% per species) and yet these two genera are anamorphs of Clavicipitales and Eurotiales like *Aschersonia*, *Acremonium* and *Aspergillus*. More work should be done to look at these related fungi.

6.4.2 The effect of media on total fatty acids

The fatty acid composition of the five strains of *Aschersonia* were influenced by media (Chapter 6 section 6.2). From the work it is concluded that all five strains of *Aschersonia* accumulated the greatest amount of total fatty acids when grown in PDB. Of the four media PDB was an undefined media and it is possible that this contained more compounds suitable for the production of fatty acids. From the results of chapter 5 and 6 (section 5.1 and 6.4.4) it was already shown that PDB was the best for production of biomass. Working with *Mortierella alpina*, Singh & Ward (1997) [ 105 ] concluded that formation of products in fungal fermentation could be

influenced by growth morphology. Although there were differences in fatty acid amounts produced over time these are very slight (Table 6.1a, b & c) suggesting that the stationary phase had largely been reached after 14 days. *Aschersonia* strains are very slow growing as shown by earlier results. However, it would be of interest in the future to harvest material at early stages of growth and determine the TFA levels before stationary phase is reached. With the results from this work it is not possible to say whether TFA levels were lower from 0-14 days and rose or whether they were high and fell from 0-14 days. Further evidence that the stationary phase had been reached is that the dry weight of mycelium for the five strains did not alter much from 14 to 30 days (Table 6.11). Singh and Ward (1997) [ 105 ]concluded that the 'amount of lipid produced by a given microbial species depends to a great extent on the development stage of growth'. They found in their study that the mucoraceous *M. alpina* accumulated biomass rapidly during the first three days and after that stayed constant. However, for their strain of *M. alpina* they noticed that lipid turnover continued after the stationary phase was reached. This is in contrast to the results of *Aschersonia* showed in this work. It is possible that these differences are due to *Aschersonia* being the anamorph of an Ascomycete (Hypocreales: Clavicipitaceae) whereas *M. alpina* is a Zygomycete (Mucorales: Mortierellaceae).

Table 6.11. Dry weight (g/l) of five strains of *Aschersonia* in different media at 22°C.

| Strain | Media | Dry weight (g/l) on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| 3162 | PDB | 5.0± 0.03 | 5.2± 0.16 | 6.30± 0.35 |
| 5877 | | 9.0± 0.03 | 10.0± 0.06 | 11.0± 0.06 |
| 5996 | | 9.5± 0.10 | 10.0± 0.10 | 12.6± 0.58 |
| 5269 | | 5.2± 0.03 | 6.1± 0.14 | 7.10± 0.28 |
| 5164 | | 9.3± 0.31 | 10.0± 0.08 | 12.2± 0.10 |
| | | | | |
| 3162 | MSM | 1.1± 0.10 | 2.9± 0.06 | 3.3± 0.22 |
| 5877 | | 3.3± 0.35 | 4.1± 0.06 | 4.5± 0.08 |
| 5996 | | 3.2± 0.14 | 3.9± 0.14 | 4.1± 0.05 |
| 5269 | | 1.5± 0.12 | 2.5± 0.07 | 3.1± 0.10 |
| 5164 | | 2.4± 0.38 | 3.7± 0.23 | 4.7± 0.32 |
| | | | | |
| 3162 | PYGM | 6.0± 0.01 | 7.0.± 0.03 | 8.2± 0.08 |
| 5877 | | 6.9± 0.12 | 7.5± 0.07 | 7.7± 0.15 |
| 5996 | | 5.7± 0.31 | 6.1± 0.03 | 6.6± 0.13 |
| 5269 | | 2.5± 0.23 | 3.8± 0.13 | 4.9± 0.21 |
| 5164 | | 6.5± 0.71 | 7.3± 0.16 | 8.9± 0.17 |
| | | | | |
| 3162 | SDB | 5.4± 0.28 | 5.6± 0.38 | 5.8± 0.33 |
| 5877 | | 5.2± 0.08 | 5.2± 0.33 | 6.6± 0.54 |
| 5996 | | 4.5± 0.23 | 5.5± 0.71 | 5.7± 0.82 |
| 5269 | | 3.5± 0.46 | 4.6± 0.54 | 5.5± 0.21 |
| 5164 | | 9.6± 0.63 | 10.5± 1.44 | 10.8± 1.72 |

6.4.3 The effect of media on individual fatty acids

The results of the work showed that the choice of media affected the quality of fatty acids produced by the five strains of *Aschersonia*. As PDB was shown to be a good producer of overall total fatty acids and was good at producing biomass, it is interesting to see if it was good for any particular fatty acids. There was no consistent pattern although for some strains there were individual observations. For *A. hypocreoidea*-5269 PDB consistently produced more oleic acid (7-10%) than the other three media (Table 6.4a). However, this did not seem to be at the expense of any one other fatty acid but at the expense of all three. Similarly, for *A. tubulata*-5877 PDB produced consistently more (saturated) palmitic acid than the other three media and this seemed to be mostly at the expense of (unsaturated) oleic acid production. For *A. tubulata*-5996 PDB appeared to be especially good for the overall production of linoleic acid (Table 6.5a) and this seemed to be largely at the expense of oleic acid (Table 6.3a) although stearic acid levels seemed to be lower as well (Table 6.3a). From this it is not possible to conclude that PDB was good for saturated or unsaturated fatty acids. There is evidence that nitrogen content of the medium affects the proportion of saturated to unsaturated fatty acids produced by many microorganisms. The nitrogen quality of media appears to affect different organisms with respect to the proportion of polyunsaturated fatty acids produced. Whereas Ben-Amotz *et. al.* (1985) [ 106 ] found that nitrogen-depleted media was good for producing higher percentages of EPA in *Botryococcus* and *Dunaliella* spp. the proportion of polyunsaturated fatty acids in the freshwater algae *Scenedesmus* and *Chlorella* increased with high nitrogen concentrations [ 107 ] as it did also for the

diatom *Phaeodactylum tricornutum* [ 108 ]. In my study, however, the effect of nitrogen was not studied but in future this could be an area for further research. In this work it is found that *Aschersonia* MSM was generally poor for the production of biomass as well as total fatty acids. However, for strains *A. hypocreoidea*-3162 and *A. tubulata*-5996 MSM appeared to stimulate the production of palmitic acid at the expense of linoleic acid. Furthermore for *A. tubulata*-5996 stearic acid seemed to be produced more when grown in MSM – at least over days 14 and 21. Again, for *A. tubulata*-5996 this was at the expense of linoleic acid which was particularly low on MSM at 14 and 21 days compared to the other three media (Table 6.5a).

6.4.4. The effect of temperatures on fatty acids

The results showed all of the five strains of *Aschersonia* accumulated higher levels of total fatty acids at 22°C than at 15 or 20°C (Table 6.6a). The other work (Chapter 6 section 6.3) showed that 22°C was also the best temperature for the production of biomass (Table 6.12). Although temperature did not seem to have a big effect on the overall levels of palmitic acid there was a notable exception with respect to *A. tubulata*. For *A. tubulata*-5877 22°C was particularly good for the production of palmitic acid compared with 15 and 20°C at 14 and 21 days (Table 6.7a). This increase in palmitic acid seemed to be mostly at the expense of linoleic acid levels (Table 6.10a). However, after 30 days the levels of palmitic acid were reduced for *A. tubulata*-5877 compared with 15 and 20°C and once more this reduction was due to an increase in the linoleic acid. In contrast, for *A. tubulata*-5996 palmitic acid levels were at their highest at 15°C and 14 days incubation and again this was at the

expense of the linoleic acid. For oleic acid the most significant observation was that for *A. tubulata*-5996 22°C resulted in much reduced levels of oleic acid (Table 16a). Interestingly, this reduction in oleic acid was because of higher levels of linoleic acid being produced (Table 6.10a). The linoleic acid levels for *A. tubulata*-5996 at 22°C were especially high and as well as being at the expense of oleic acid the levels of palmitic and stearic acid were also reduced. *A. tubulata*-5996 also showed significant temperature effects after 30 days. For example at 20°C the oleic acid level was at its highest after 30 days and again this seemed to be at the expense of linoleic acid. Sumner, Morgan & Evans (1969) [ 109 ] noted that there 'are conflicting reports in the literature on the effect of environmental temperature on the composition of the fatty acids of fungal lipids'. Thirty years on this is even more the case as more studies have been done on a greater range of fungi. In this study it is clear that even strains within species can give a different response.

Table 6.12. Dry weight (g/l) of five strains of *Aschersonia* grown at different temperatures in PDB.

| Strain | Temperature (°C) | Dry weight (g/l) on | | |
|---|---|---|---|---|
| | | 14 days | 21 days | 30 days |
| 3162 | 15 | 1.5± 0.10 | 2.9± 0.62 | 3.7± 0.24 |
| 5877 | | 5.2± 0.18 | 6.6± 0.29 | 7.2± 0.18 |
| 5996 | | 5.0± 0.45 | 6.3± 0.34 | 7.5± 0.35 |
| 5269 | | 2.4± 0.42 | 3.6± 0.36 | 5.0± 0.31 |
| 5164 | | 5.7± 0.25 | 6.8± 0.37 | 8.0± 0.19 |
| | | | | |
| 3162 | 20 | 7.2± 0.23 | 8.1± 0.12 | 8.7± 0.33 |
| 5877 | | 9.6± 0.30 | 11.0± 0.15 | 11.6± 0.33 |
| 5996 | | 10.3± 0.22 | 11.7± 0.33 | 12.6± 0.40 |
| 5269 | | 6.4± 0.25 | 7.3± 0.35 | 8.3± 0.30 |
| 5164 | | 10.3± 0.31 | 11.4± 0.21 | 12.6± 0.38 |
| | | | | |
| 3162 | 22 | 7.7± 0.27 | 8.6± 0.28 | 9.1± 0.14 |
| 5877 | | 10.2± 0.20 | 11.6± 0.30 | 12.7± 0.25 |
| 5996 | | 10.7± 0.30 | 12.2± 0.36 | 13.0± 0.06 |
| 5269 | | 6.5± 0.31 | 7.6± 0.38 | 8.4± 0.36 |
| 5164 | | 10.7± 0.50 | 11.8± 0.28 | 12.9± 0.33 |

# Chapter 7

# Conclusions

## 7.1. General conclusions

Although the genus *Aschersonia* has been considered for biocontrol in the past and more recently there is very little literature apart from taxonomic work. The results from this work represent the first attempt to understand more about the genus and will form a base for further study.

## 7.2. Specific conclusions

### 7.2.1 The effect of nutrient quality and temperature on morphology

Early taxonomists such as Petch and Mains attached importance to conidial size especially. This work has shown that both media and temperature have an effect on the size of conidia produced by *Aschersonia* strains in culture. Care must be taken in interpreting spore size for specimens collected in the field. The field collector can have no knowledge of how nutrient-rich or poor was the host scale insect and little knowledge of the temperatures operating at the time of infection. Although scale insects can not move this study does suggest that the elevation of the forest may affect the spore size and this is still reflected in the spore size found in culture.

### 7.2.2 The effect of nutrient quality and temperature on fatty acids

The analysis of fatty acid profiles has been increasingly used for organisms with few morphological characters such as yeasts and bacteria [ 110 ]. Although only five strains were used in this preliminary study it has become clear that even within one species there can be significant differences in the fatty acid profiles. It is clear that many more strains of a given species should be examined from different regions so that a picture of fatty acid profiles for certain species can be developed. The result has shown that for *Aschersonia* media and temperature affect the fatty acid profiles. If fatty acids are to be used in helping taxonomists then the conditions must be clearly stated. Although PDA proved to be the best media for growth and generally for fatty acid production it may not be the best media for helping understand taxonomic or geographic differences. As it is an undefined medium there are possibly many nutrients that different strains can use differently. In the future it may be better to use one of the poorer media such as MSM or SDA since these gave more variable responses for strains with some strains being able to make use of the media while others could not.

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Appendix A.

Table A.1 Fatty Acid Composition of *Aschersonia placenta* 5164 in various media at 22°C

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 22 | 14 | 18.71± 0.04 | 5.01± 0.04 | 27.03 ± 0.12 | 49.26 ± 0.11 | 1.22 ± 0.02 |
| | | 21 | 17.57 ± 0.40 | 6.34 ± 0.15 | 25.69 ± 0.18 | 50.41 ± 0.43 | 1.17 ± 0.52 |
| | | 30 | 18.97 ± 1.00 | 7.96 ± 0.26 | 28.37 ± 0.57 | 44.86 ± 1.87 | 1.33 ± 0.01 |
| SDB | 22 | 14 | 16.90 ± 0.09 | 6.54 ± 0.64 | 22.96 ± 1.08 | 53.61 ± 0.53 | 1.06 ± 0.04 |
| | | 21 | 15.55 ± 0.24 | 4.36 ± 0.02 | 24.67 ± 0.13 | 55.4 ± 0.13 | 1.04 ± 0.22 |
| | | 30 | 14.44 ± 0.13 | 5.75 ± 0.10 | 26.55 ± 0.06 | 53.27 ± 0.02 | 1.04 ± 0.04 |
| PYGM | 22 | 14 | 20.54 ± 0.35 | 5.27 ± 0.03 | 24.49 ± 0.13 | 49.71 ± 0.50 | 0.88 ± 0.02 |
| | | 21 | 19.58 ± 0.26 | 6.50± 0.51 | 25.64 ± 0.18 | 48.29 ± 0.07 | 0.98 ± 0.04 |
| | | 30 | 21.04 ± 0.35 | 7.40 ± 0.13 | 26.70 ± 0.71 | 44.96 ± 0.62 | 0.94 ± 0.01 |
| MSM | 22 | 14 | 15.57 ± 0.30 | 7.54 ± 0.22 | 26.45 ± 0.37 | 50.45 ± 0.45 | 0.97 ± 0.08 |
| | | 21 | 14.43 ± 0.34 | 6.76 ± 0.72 | 29.36 ± 0.45 | 49.46 ± 0.83 | 0.89 ± 0.01 |
| | | 30 | 14.58 ± 1.70 | 6.64 ± 0.19 | 33.01± 0.47 | 45.77 ± 1.05 | 0.93 ± 0.03 |

Table A.2 Fatty Acid Composition of *Aschersonia tubulata* 5996 in various media at 22°C

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 22 | 14 | 18.38 ± 0.33 | 5.35 ± 0.36 | 9.97 ± 0.38 | 66.04 ± 0.42 | 1.36 ± 0.65 |
| | | 21 | 19.70 ± 0.18 | 3.38 ± 0.45 | 8.43 ± 0.64 | 67.32 ± 1.16 | 1.45 ± 0.07 |
| | | 30 | 17.55 ± 1.04 | 3.73 ± 0.45 | 6.27 ± 0.28 | 71.99 ± 1.24 | 1.26 ± 0.02 |
| SDB | 22 | 14 | 24.08 ± 0.57 | 7.65 ± 0.23 | 24.74 ± 0.16 | 43.54 ± 0.18 | 0.42 ± 0.02 |
| | | 21 | 21.76 ± 0.33 | 9.08 ± 0.23 | 31.25 ± 0.18 | 37.55 ± 0.19 | 0.76 ± 0.07 |
| | | 30 | 21.84 ± 1.90 | 7.50 ± 1.16 | 34.51 ± 0.61 | 36.15 ± 0.14 | 0.58 ± 0.02 |
| PYGM | 22 | 14 | 16.24 ± 0.35 | 6.14 ± 0.23 | 17.93 ± 0.08 | 59.70 ± 0.51 | 0.52 ± 0.07 |
| | | 21 | 18.48 ± 0.09 | 4.35 ± 0.24 | 19.98 ± 0.08 | 57.20 ± 0.08 | 0.48 ± 0.02 |
| | | 30 | 17.16 ± 0.07 | 5.30 ± 0.40 | 19.44 ± 0.30 | 58.11 ± 0.16 | 0.43 ± 0.05 |
| MSM | 22 | 14 | 28.45 ± 0.45 | 10.51 ± 0.79 | 22.07 ± 0.19 | 38.98 ± 1.43 | 0.97 ± 0.08 |
| | | 21 | 32.12 ± 0.69 | 10.15 ± 0.44 | 16.89 ± 0.23 | 40.85 ± 0.02 | 0.85 ± 0.02 |
| | | 30 | 22.24 ± 0.90 | 4.87 ± 0.43 | 16.61 ± 0.13 | 56.25 ± 0.28 | 0.77 ± 0.03 |

Table A.3 Fatty Acid Composition of *Aschersonia tubulata* 5877 in various media at 22°C

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 22 | 14 | 24.85 ± 0.27 | 6.25 ± 0.33 | 18.11± 0.22 | 50.37 ± 0.29 | 1.82 ± 0.02 |
| | | 21 | 26.11 ± 0.64 | 5.74 ± 0.06 | 17.82 ± 0.09 | 49.77 ± 0.74 | 1.75 ± 0.05 |
| | | 30 | 22.74 ± 0.29 | 7.05 ± 0.09 | 18.69 ± 0.78 | 51.53 ± 0.98 | 1.50 ± 0.04 |
| SDB | 22 | 14 | 17.41 ± 0.37 | 7.22 ± 0.17 | 28.42 ± 0.30 | 46.16 ± 0.23 | 1.73 ± 0.02 |
| | | 21 | 15.38 ± 0.48 | 6.68 ± 0.01 | 26.59 ± 0.15 | 51.50 ± 0.13 | 1.63 ± 0.03 |
| | | 30 | 17.39 ± 0.10 | 3.73 ± 0.11 | 20.00 ± 0.45 | 58.89 ± 0.45 | 1.52 ± 0.02 |
| PYGM | 22 | 14 | 18.32 ± 1.64 | 3.20 ± 0.85 | 19.62 ± 0.70 | 58.77 ± 0.04 | 1.21 ± 0.03 |
| | | 21 | 19.29 ± 0.30 | 4.18 ± 0.03 | 20.35 ± 0.24 | 56.19 ± 0.09 | 1.04 ± 0.09 |
| | | 30 | 19.61 ± 0.27 | 4.07 ± 0.03 | 15.40 ± 0.01 | 60.58 ± 0.28 | 0.97 ± 0.08 |
| MSM | 22 | 14 | 19.29 ± 0.08 | 8.70 ± 0.06 | 27.22 ± 0.37 | 44.80 ± 0.35 | 0.87 ± 0.06 |
| | | 21 | 18.80 ± 0.06 | 5.47 ± 0.30 | 25.23 ± 0.26 | 50.51 ± 0.01 | 0.84 ± 0.09 |
| | | 30 | 19.57 ± 0.08 | 4.62 ± 0.06 | 23.36 ± 0.30 | 52.47 ± 0.45 | 0.77 ± 0.07 |

Table A.4 Fatty Acid Composition of *Aschersonia hypocreoidea* 3162 in various media at 22°C

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 22 | 14 | 17.43 ± 0.01 | 5.82 ± 0.42 | 16.05 ± 0.35 | 60.36 ± 0.82 | 1.09 ± 0.15 |
| | | 21 | 19.78 ± 0.35 | 4.43 ± 0.16 | 16.97 ± 0.44 | 58.33 ± 0.75 | 1.07 ± 0.06 |
| | | 30 | 19.85 ± 0.06 | 5.40 ± 0.39 | 15.40 ± 0.01 | 59.36 ± 0.32 | 1.04 ± 0.02 |
| SDB | 22 | 14 | 18.32 ± 0.13 | 4.47 ± 0.06 | 25.07 ± 0.06 | 52.15 ± 0.13 | 0.81 ± 0.12 |
| | | 21 | 21.27 ± 0.21 | 5.36 ± 0.17 | 22.17 ± 0.13 | 51.21 ± 0.09 | 0.80 ± 0.02 |
| | | 30 | 19.48 ± 0.06 | 5.35 ± 0.11 | 24.03 ± 0.07 | 51.26 ± 0.18 | 0.80 ± 0.11 |
| PYGM | 22 | 14 | 16.58 ± 0.09 | 7.35 ± 0.08 | 16.85 ± 0.16 | 59.23 ± 0.33 | 0.92 ± 0.05 |
| | | 21 | 15.33 ± 0.28 | 6.56 ± 0.19 | 17.37 ± 0.28 | 60.75 ± 0.76 | 0.97 ± 0.08 |
| | | 30 | 17.77 ± 0.14 | 7.67 ± 0.23 | 18.31 ± 0.24 | 56.25 ± 0.61 | 0.90 ± 0.08 |
| MSM | 22 | 14 | 24.66 ± 0.73 | 5.76 ± 0.90 | 20.47 ± 1.29 | 48.83 ± 3.30 | 0.79 ± 0.16 |
| | | 21 | 25.77 ± 0.08 | 6.66 ± 0.35 | 20.77 ± 0.86 | 46.81 ± 0.44 | 0.77 ± 0.05 |
| | | 30 | 23.23 ± 0.79 | 5.24 ± 0.09 | 20.68 ± 0.27 | 50.85 ± 0.44 | 0.77 ± 0.04 |

Table A.5 Fatty Acid Composition of *Aschersonia hypocreoidea* 5269 in various media at 22°C

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 22 | 14 | 15.43 ± 0.12 | 5.22 ± 0.06 | 28.23 ± 0.16 | 51.14 ± 0.22 | 0.87 ± 0.16 |
| | | 21 | 16.09 ± 0.10 | 5.82 ± 0.55 | 27.47 ± 0.66 | 50.63 ± 1.11 | 0.91 ± 0.01 |
| | | 30 | 15.85 ± 0.34 | 6.10 ± 0.11 | 28.39 ± 0.60 | 49.83 ± 0.06 | 0.94 ± 0.28 |
| SDB | 22 | 14 | 17.34 ± 0.32 | 6.55 ± 0.46 | 20.18 ± 0.37 | 55.94 ± 0.51 | 0.88 ± 0.07 |
| | | 21 | 18.78 ± 0.31 | 6.94 ± 0.38 | 19.22 ± 0.88 | 55.07 ± 1.58 | 0.91 ± 0.08 |
| | | 30 | 19.28 ± 0.23 | 7.58 ± 0.14 | 18.57 ± 0.09 | 54.58 ± 0.46 | 0.91 ± 0.01 |
| PYGM | 22 | 14 | 20.36 ± 0.22 | 7.71 ± 0.39 | 18.06 ± 0.09 | 53.88 ± 0.09 | 0.79 ± 0.05 |
| | | 21 | 19.32 ± 0.28 | 8.69 ± 0.40 | 20.70 ± 0.94 | 51.30 ± 1.62 | 0.82 ± 0.04 |
| | | 30 | 21.12 ± 0.09 | 7.49 ± 0.54 | 22.83 ± 0.07 | 48.57 ± 0.56 | 0.85 ± 0.06 |
| MSM | 22 | 14 | 18.25 ± 1.03 | 8.64 ± 0.33 | 20.72 ± 0.09 | 52.40 ± 0.80 | 0.75 ± 0.04 |
| | | 21 | 19.51 ± 0.41 | 7.29 ± 0.08 | 22.48 ± 0.16 | 50.73 ± 0.65 | 0.77 ± 0.03 |
| | | 30 | 17.05 ± 1.06 | 6.66 ± 0.79 | 23.42 ± 0.28 | 52.88 ± 0.01 | 0.78 ± 0.02 |

Table A.6 Fatty Acid Composition of *Aschersonia hypocreoidea* 5269 in PDB at Different Temperature

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 15 | 14 | 17.62 ± 0.20 | 6.52 ± 0.26 | 26.58 ± 0.52 | 49.28 ± 0.07 | 0.86 ± 0.02 |
| | | 21 | 18.61 ± 0.12 | 6.54 ± 0.59 | 26.95 ± 0.21 | 47.91 ± 0.68 | 0.91 ± 0.07 |
| | | 30 | 16.26 ± 0.35 | 6.13 ± 0.40 | 26.26 ± 0.40 | 51.36 ± 0.45 | 0.94 ± 0.04 |
| | 20 | 14 | 14.67 ± 0.32 | 6.24 ± 0.56 | 27.52 ± 0.42 | 51.59 ± 0.46 | 1.11 ± 0.04 |
| | | 21 | 15.51 ± 0.09 | 5.51 ± 0.41 | 28.70 ± 0.40 | 50.29 ± 0.71 | 1.14 ± 0.02 |
| | | 30 | 15.62 ± 0.70 | 6.11 ± 0.01 | 27.99 ± 0.07 | 50.29 ± 0.78 | 1.16 ± 0.0.3 |
| | 22 | 14 | 15.29 ± 0.45 | 5.44 ± 0.26 | 28.56 ± 0.13 | 50.72 ± 0.06 | 1.23 ± 0.01 |
| | | 21 | 15.78 ± 0.51 | 5.64 ± 0.51 | 27.96 ± 0.23 | 50.63 ± 0.23 | 1.25 ± 0.03 |
| | | 30 | 15.99 ± 0.18 | 5.53 ± 0.70 | 28.20 ± 0.32 | 50.29 ± 0.84 | 1.33 ± 0.04 |

Table A.7 Fatty Acid Composition of *Aschersonia hypocreoidea* 3162 in PDB at Different Temperature

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 15 | 14 | 19.16 ± 0.09 | 5.39 ± 0.24 | 17.41 ± 0.09 | 58.05 ± 0.24 | 0.95 ± 0.04 |
| | | 21 | 20.24 ± 0.06 | 6.53 ± 0.26 | 18.01 ± 0.73 | 55.24 ± 0.40 | 0.83 ± 0.02 |
| | | 30 | 19.35 ± 0.04 | 7.44 ± 0.31 | 18.21 ± 0.65 | 55.01 ± 0.38 | 0.77 ± 0.26 |
| | 20 | 14 | 19.66 ± 0.12 | 6.52 ± 0.33 | 18.75 ± 0.17 | 55.08 ± 0.04 | 0.86 ± 0.04 |
| | | 21 | 20.67 ± 0.17 | 7.38 ± 0.09 | 17.65 ± 0.37 | 54.31 ± 0.11 | 0.77 ± 0.06 |
| | | 30 | 22.13 ± 0.60 | 7.23 ± 0.32 | 20.55 ± 0.17 | 49.42 ± 0.17 | 0.86 ± 0.07 |
| | 22 | 14 | 18.80 ± 0.01 | 5.20 ± 0.24 | 18.24 ± 0.24 | 57.36 ± 0.01 | 0.94 ± 0.03 |
| | | 21 | 19.09 ± 0.31 | 4.55 ± 0.23 | 17.32 ± 0.11 | 59.04 ± 0.03 | 1.07 ± 0.08 |
| | | 30 | 20.01 ± 0.28 | 5.34 ± 0.08 | 16.59 ± 0.05 | 58.08 ± 0.31 | 1.01 ± 0.04 |

Table A.8 Fatty Acid Composition of *Aschersonia tubulata* 5877 in PDB at Different Temperature

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 15 | 14 | 18.95 ± 0.13 | 6.05 ± 0.52 | 18.43 ± 0.64 | 56.58 ± 1.29 | 0.88 ± 0.01 |
| | | 21 | 16.72 ± 0.74 | 6.30 ± 0.04 | 19.02 ± 0.98 | 57.99 ± 1.74 | 0.91 ± 0.02 |
| | | 30 | 20.04 ± 0.23 | 7.56 ± 0.12 | 19.08 ± 0.66 | 53.31 ± 0.55 | 0.97 ± 0.03 |
| | 20 | 14 | 21.81 ± 0.10 | 5.89 ± 0.63 | 19.02 ± 0.53 | 53.29 ± 1.26 | 1.71 ± 0.25 |
| | | 21 | 23.57 ± 0.22 | 8.49 ± 1.12 | 19.43 ± 0.42 | 48.52 ± 0.48 | 1.67 ± 0.08 |
| | | 30 | 23.04 ± 0.14 | 7.72 ± 0.06 | 19.49 ± 0.14 | 49.76 ± 0.06 | 1.52 ± 0.25 |
| | 22 | 14 | 24.80 ± 0.53 | 6.65 ± 0.19 | 18.33 ± 0.06 | 50.24 ± 0.66 | 1.82 ± 0.23 |
| | | 21 | 26.18 ± 0.44 | 5.97 ± 0.25 | 17.93 ± 0.05 | 49.93 ± 0.74 | 1.76 ± 0.04 |
| | | 30 | 21.39 ± 0.13 | 6.77 ± 0.06 | 18.62 ± 0.19 | 53.23 ± 0.38 | 1.64 ± 0.25 |

Table A.9 Fatty Acid Composition of *Aschersonia tubulata* 5996 in PDB at Different Temperature

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 15 | 14 | 26.40 ± 0.74 | 6.14 ± 0.10 | 15.06 ± 0.11 | 52.41± 0.54 | 1.11 ± 0.03 |
| | | 21 | 21.49 ± 0.49 | 8.01 ± 0.20 | 11.88 ± 0.42 | 58.63 ± 0.27 | 0.97 ± 0.25 |
| | | 30 | 18.45 ± 0.74 | 7.73 ± 0.33 | 9.57 ± 0.15 | 64.25 ± 1.20 | 0.88 ± 0.08 |
| | 20 | 14 | 19.71 ± 0.18 | 6.35 ± 0.24 | 15.46 ± 0.40 | 58.49 ± 0.46 | 1.07 ± 0.05 |
| | | 21 | 22.21 ± 0.47 | 6.39 ± 0.08 | 11.62 ± 0.57 | 59.79 ± 0.18 | 1.16 ± 0.04 |
| | | 30 | 19.39 ± 0.06 | 5.82 ± 0.11 | 17.59 ± 0.31 | 57.21 ± 0.47 | 1.25 ± 0.07 |
| | 22 | 14 | 19.28 ± 0.78 | 4.96 ± 0.26 | 9.57 ± 0.85 | 66.20 ± 1.89 | 1.29 ± 0.02 |
| | | 21 | 19.43 ± 0.30 | 4.24 ± 0.11 | 8.99 ± 0.18 | 67.34 ± 0.01 | 1.41 ± 0.06 |
| | | 30 | 18.34 ± 0.50 | 4.49 ± 0.13 | 7.80 ± 0.21 | 69.39 ± 0.43 | 1.30 ± 0.08 |

Table A.10 Fatty Acid Composition of *Aschersonia placenta* 5164 in PDB at Different Temperature

| Media | Temperature (°C) | Time (d) | Fatty Acid Composition | | | | % Total Fatty Acid |
|---|---|---|---|---|---|---|---|
| | | | C 16:0 | C 18:0 | C18:1 | C 18:2 | |
| PDB | 15 | 14 | 16.09 ± 0.07 | 3.56 ± 0.07 | 23.41 ± 0.21 | 56.95 ± 0.99 | 0.95 ± 0.02 |
| | | 21 | 15.90 ± 0.04 | 4.55 ± 1.41 | 23.72 ± 0.34 | 55.84 ± 0.27 | 0.92 ± 0.25 |
| | | 30 | 17.77 ± 0.21 | 4.91 ± 0.08 | 22.02 ± 0.25 | 55.31 ± 0.04 | 0.86 ± 0.05 |
| | 20 | 14 | 16.56 ± 0.04 | 5.45 ± 0.15 | 25.54 ± 2.46 | 52.46 ± 1.41 | 1.18 ± 0.14 |
| | | 21 | 17.73 ± 0.34 | 4.56 ± 0.18 | 27.65 ± 0.30 | 50.06 ± 0.82 | 1.18 ± 0.06 |
| | | 30 | 16.28 ± 0.49 | 5.56 ± 0.21 | 31.29 ± 0.05 | 46.93 ± 0.58 | 1.03 ± 0.07 |
| | 22 | 14 | 18.66 ± 0.14 | 4.90 ± 2.69 | 26.92 ± 3.65 | 49.53 ± 0.88 | 1.18 ± 0.11 |
| | | 21 | 18.06 ± 0.19 | 6.61 ± 0.12 | 27.75 ± 0.33 | 47.59 ± 0.41 | 1.34 ± 0.15 |
| | | 30 | 18.63 ± 0.30 | 7.51 ± 1.45 | 28.62 ± 0.92 | 45.40 ± 2.15 | 1.20 ± 0.09 |

Appendix B.

Table B.1 Dry weight of five strains of *Aschersonia* in different media (for percent fatty acids as percent dry weight).

| Species | Code | Media | Temp | Age (days) | Dw (grams) |
|---|---|---|---|---|---|
| *A.. hypocreoidea* | 5269 | PDB | 22 | 14 | 0.83 |
| | | | | | 0.82 |
| | | | | 21 | 1.12 |
| | | | | | 1.12 |
| | | | | 30 | 1.13 |
| | | | | | 1.13 |
| *A.. hypocreoidea* | 5269 | MSM | 22 | 14 | 0.75 |
| | | | | | 0.77 |
| | | | | 21 | 0.80 |
| | | | | | 0.80 |
| | | | | 30 | 0.71 |
| | | | | | 0.71 |
| *A. hypocreoidea* | 5269 | PYGM | 22 | 14 | 0.68 |
| | | | | | 0.68 |
| | | | | 21 | 0.70 |
| | | | | | 0.70 |
| | | | | 30 | 0.71 |
| | | | | | 0.71 |
| *A. hypocreoidea* | 5269 | SDB | 22 | 14 | 0.51 |
| | | | | | 0.52 |
| | | | | 21 | 0.62 |
| | | | | | 0.63 |
| | | | | 30 | 0.63 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 0.63 |
| *A. hypocreoidea* | 3162 | PDB | 22 | 14 | 0.65 |
| | | | | | 0.60 |
| | | | | 21 | 0.53 |
| | | | | | 0.53 |
| | | | | 30 | 0.50 |
| | | | | | 0.50 |
| *A. hypocreoidea* | 3162 | MSM | 22 | 14 | 0.43 |
| | | | | | 0.47 |
| | | | | 21 | 0.48 |
| | | | | | 0.50 |
| | | | | 30 | 0.41 |
| | | | | | 0.48 |
| *A. hypocreoidea* | 3162 | PYGM | 22 | 14 | 0.80 |
| | | | | | 0.81 |
| | | | | 21 | 0.81 |
| | | | | | 0.82 |
| | | | | 30 | 0.80 |
| | | | | | 0.80 |
| *A. hypocreoidea* | 3162 | SDB | 22 | 14 | 0.55 |
| | | | | | 0.55 |
| | | | | 21 | 0.52 |
| | | | | | 0.54 |
| | | | | 30 | 0.53 |
| | | | | | 0.53 |
| | | | | 14 | 1.09 |
| *A. tubulata* | 5877 | PDB | 22 | | 1.10 |
| | | | | 21 | 0.90 |
| | | | | | 0.90 |
| | | | | 30 | 1.10 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 1.09 |
| *A. tubulata* | 5877 | MSM | 22 | 14 | 0.51 |
| | | | | | 0.50 |
| | | | | 21 | 0.49 |
| | | | | | 0.49 |
| | | | | 30 | 0.41 |
| | | | | | 0.41 |
| *A. tubulata* | 5877 | PYGM | 22 | 14 | 0.90 |
| | | | | | 0.90 |
| | | | | 21 | 0.85 |
| | | | | | 0.85 |
| | | | | 30 | 0.81 |
| *A.tubulata* | 5877 | SDB | 22 | | 0.81 |
| | | | | 14 | 0.62 |
| | | | | | 0.61 |
| | | | | 21 | 0.55 |
| | | | | | 0.54 |
| | | | | 30 | 0.52 |
| | | | | | 0.52 |
| *A. tubulata* | 5996 | PDB | 22 | 14 | 1.00 |
| | | | | | 0.99 |
| | | | | 21 | 1.30 |
| | | | | | 1.22 |
| | | | | 30 | 0.94 |
| | | | | | 0.96 |
| *A. tubulata* | 5996 | MSM | 22 | 14 | 0.65 |
| | | | | | 0.65 |
| | | | | 21 | 0.60 |
| | | | | | 0.59 |
| | | | | 30 | 0.51 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 0.51 |
| | | | | | |
| *A. tubulata* | 5996 | PYGM | 22 | 14 | 0.79 |
| | | | | | 0.79 |
| | | | | 21 | 0.71 |
| | | | | | 0.71 |
| | | | | 30 | 0.70 |
| | | | | | 0.70 |
| *A. tubulata* | 5996 | SDB | 22 | 14 | 0.46 |
| | | | | | 0.47 |
| | | | | | 0.50 |
| | | | | 21 | 0.50 |
| | | | | | 0.52 |
| | | | | 30 | 0.52 |
| *A. placenta* | 5164 | PDB | 22 | 14 | 1.21 |
| | | | | | 1.21 |
| | | | | 21 | 1.20 |
| | | | | | 1.00 |
| | | | | 30 | 1.32 |
| | | | | | 1.06 |
| *A. placenta* | 5164 | MSM | 22 | 14 | 0.78 |
| | | | | | 0.74 |
| | | | | 21 | 0.66 |
| | | | | | 0.64 |
| | | | | 30 | 0.71 |
| | | | | | 0.76 |
| *A. placenta* | 5164 | PYGM | 22 | 14 | 0.77 |
| | | | | | 0.70 |
| | | | | 21 | 0.90 |
| | | | | | 0.90 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | 30 | 0.84 |
| | | | | | 0.84 |
| *A. placenta* | | | | 14 | 0.88 |
| | | | | | 0.88 |
| | | | | 21 | 0.93 |
| | 5164 | SDB | 22 | | 0.95 |
| | | | | 30 | 0.92 |
| | | | | | 0.92 |

Table B.2 Dry weight of five strains of *Aschersonia* at different temperature (for percent fatty acids as percent dry weight).

| Species | Strains | Temp (°C) | Media | Age (days) | Dw (grams) |
|---|---|---|---|---|---|
| *A. tubulata* | 5996 | 15 | | 14 | 0.85 |
| | | | | | 0.84 |
| | | | | | 1.12 |
| | | | | 21 | 0.97 |
| | | | | | 0.87 |
| | | | | | 0.96 |
| | | | | 30 | 0.87 |
| | | | | | 0.88 |
| | | | | | 0.89 |
| *A. tubulata* | 5996 | 20 | | 14 | 1.12 |
| | | | | | 1.11 |
| | | | | | 1.02 |
| | | | | 21 | 1.24 |
| | | | | | 1.15 |
| | | | | | 1.26 |
| | | | | 30 | 1.21 |
| | | | | | 1.22 |
| | | | | | 1.11 |
| *A. tubulata* | 5996 | 22 | | 14 | 1.28 |
| | | | | | 1.29 |
| | | | | | 1.31 |
| | | | | 21 | 1.41 |
| | | | | | 1.42 |
| | | | | | 1.4 |
| | | | | 30 | 1.31 |
| | | | | | 1.28 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 1.29 |
| *A. tubulata* | 5877 | 15 | | 14 | 0.88 |
| | | | | | 0.87 |
| | | | | | 0.88 |
| | | | | 21 | 0.89 |
| | | | | | 0.91 |
| | | | | | 0.92 |
| | | | | 30 | 0.97 |
| | | | | | 0.98 |
| | | | | | 0.97 |
| *A. tubulata* | 5877 | 20 | | 14 | 1.72 |
| | | | | | 1.71 |
| | | | | | 1.7 |
| | | | | 21 | 1.68 |
| | | | | | 1.66 |
| | | | | | 1.66 |
| | | | | 30 | 1.52 |
| | | | | | 1.5 |
| | | | | | 1.51 |
| *A. tubulata* | 5877 | 22 | | 14 | 1.83 |
| | | | | | 1.82 |
| | | | | | 1.81 |
| | | | | 21 | 1.76 |
| | | | | | 1.75 |
| | | | | | 1.75 |
| | | | | 30 | 1.64 |
| | | | | | 1.63 |
| | | | | | 1.63 |
| | | | | 14 | 0.92 |
| | | | | | 0.93 |

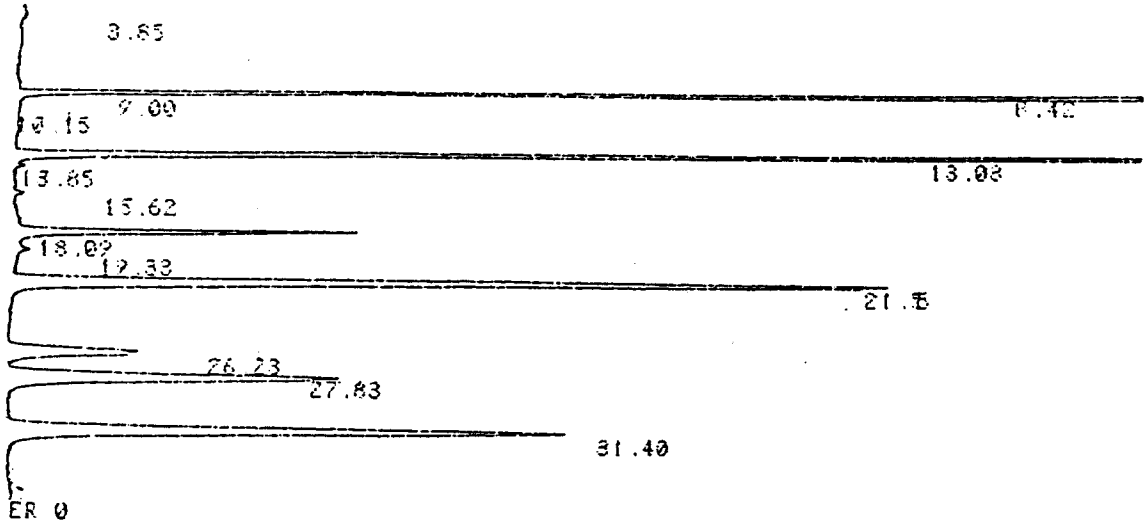
| | | | | | |
|---|---|---|---|---|---|
| | | | | | 0.91 |
| | | | | 21 | 0.87 |
| *A. placenta* | 5164 | 20 | | | 0.85 |
| | | | | | 0.84 |
| | | | | 30 | 0.96 |
| | | | | | 0.95 |
| | | | | | 0.93 |
| | | | | 14 | 0.92 |
| | | | | | 1.12 |
| | | | | | 1.13 |
| | | | | | 1.14 |
| *A. placenta* | 5164 | 20 | | 21 | 1.2 |
| | | | | | 1.22 |
| | | | | | 1.19 |
| | | | | 30 | 1.17 |
| | | | | | 1.16 |
| | | | | | 1.21 |
| | | | | 14 | 1.2 |
| | | | | | 1.19 |
| | | | | | 1.34 |
| *A. placenta* | 5164 | 22 | | 21 | 1.32 |
| | | | | | 1.33 |
| | | | | | 1.19 |
| | | | | 30 | 1.18 |
| | | | | | 1.16 |
| | | | | | 0.96 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | 14 | 0.95 |
| | | | | | 0.94 |
| | | | | | 0.84 |
| *A. hypocreoidea* | 3162 | 15 | | 21 | 0.83 |
| | | | | | 0.81 |
| | | | | | 0.76 |
| | | | | 30 | 0.79 |
| | | | | | 0.78 |
| | | | | | 0.87 |
| | | | | 14 | 0.85 |
| | | | | | 0.84 |
| | | | | | 0.76 |
| | | | | 21 | 0.79 |
| *A. hypocreoidea* | 3162 | 20 | | | 0.77 |
| | | | | | 0.84 |
| | | | | 30 | 0.85 |
| | | | | | 0.87 |
| | | | | | 0.96 |
| | | | | 14 | 0.89 |
| | | | | | 0.92 |
| | | | | | 1.08 |
| *A. hypocreoidea* | 3162 | 22 | | 21 | 1.05 |
| | | | | | 1.06 |
| | | | | | 0.98 |
| | | | | 30 | 1.02 |
| | | | | | 1.04 |
| | | | | | 0.86 |
| | | | | 14 | 0.84 |
| *A. hypocreoidea* | 5269 | 15 | | | 0.86 |
| | | | | | 0.90 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | 21 | 0.92 |
| | | | | | 0.92 |
| *A. hypocreoidea* | 5269 | 15 | | | 0.93 |
| | | | | 30 | 0.94 |
| | | | | | 0.95 |
| | | | | | 1.10 |
| | | | | 14 | 1.11 |
| | | | | | 1.11 |
| | | | | | 1.13 |
| *A. hypocreoidea* | 5269 | 20 | | 21 | 1.15 |
| | | | | | 1.15 |
| | | | | | 1.15 |
| | | | | 30 | 1.16 |
| | | | | | 1.17 |
| | | | | | 1.23 |
| | | | | 14 | 1.23 |
| | | | | | 1.22 |
| | | | | | 1.24 |
| | | | | 21 | 1.25 |
| *A. hypocreoidea* | 5269 | 22 | | | 1.26 |
| | | | | | 1.32 |
| | | | | 30 | 1.33 |
| | | | | | 1.34 |
| | | | | | 1.34 |

Appendix C.

Fig. C.1 The example of chromatogram output of *Aschersonia tubulata*- 5877 in SDB at 22°C , 14 days.



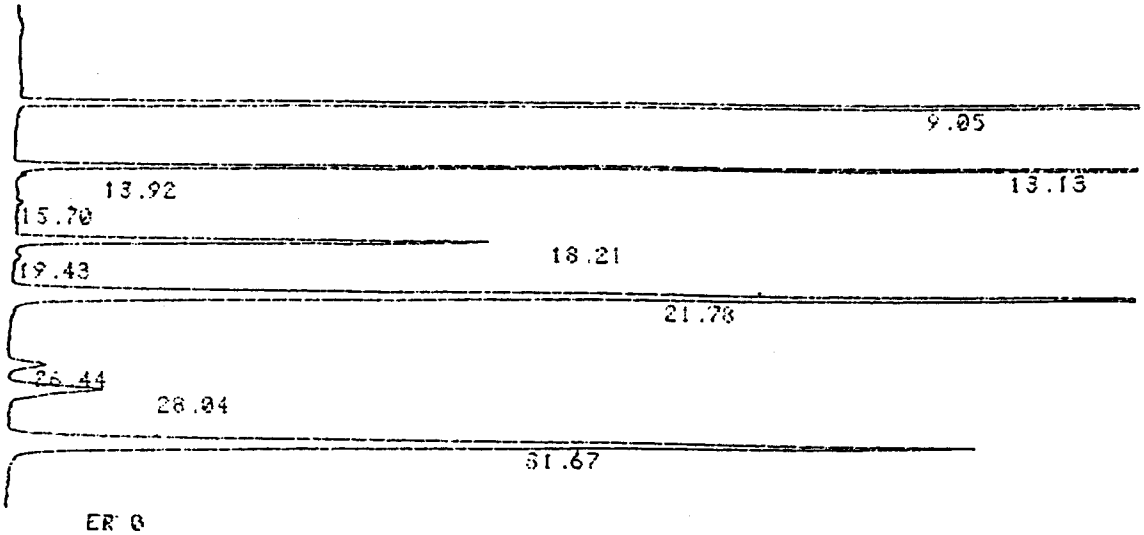

INPUT OVERRANGE AT RT= 9.05

ASC 10/07/96 11:29:28 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 2 INDEX 2 BIN 2

| PEAK# | AREA% | RT | AREA BC |
|---|---|---|---|
| 1 | 0.011 | 3.85 | 1474 01 |
| 2 | 0.003 | 8.42 | 417 02 |
| 3 | 95.578 | 9. | 12811449 02 |
| 4 | 0.004 | 10.15 | 524 03 |
| 5 | 1.461 | 13.08 | 195804 08 |
| 6 | 0.003 | 13.85 | 380 05 |
| 7 | 0.004 | 15.62 | 585 01 |
| 8 | 0.302 | 18.09 | 40532 02 |
| 9 | 0.023 | 19.33 | 3056 03 |
| 10 | 1.028 | 21.55 | 137826 01 |
| 11 | 0.187 | 26.23 | 25013 02 |
| 12 | 0.516 | 27.83 | 69227 03 |
| 13 | 0.88 | 31.4 | 117693 01 |
| TOTAL | 100. | | 13404179 |

Fig. C.2 The example of chromatogram output of *Aschersonia tubulata*- 5996 in PDB at 22°C , 30 days.




ER 0
DATA SAVED TO BIN # 8

INPUT OVERRANGE AT RT= 9.08

ASC 10/07/96 16:06:49 CH= "A" FS= 1.

FILE 1. METHOD 0. RUN 8 INDEX 0 BIN 8

| PEAK# | AREA% | RT | AREA | BC |
|---|---|---|---|---|
| 1 | 94.954 | 9.05 | 15323170 | 01 |
| 2 | 1.843 | 13.13 | 297382 | 08 |
| 3 | 0.002 | 13.92 | 385 | 05 |
| 4 | 0.003 | 15.7 | 493 | 01 |
| 5 | 0.334 | 18.21 | 53918 | 01 |
| 6 | 0.003 | 19.43 | 431 | 01 |
| 7 | 1.406 | 21.78 | 226970 | 01 |
| 8 | 0.046 | 26.44 | 7434 | 02 |
| 9 | 0.141 | 28.04 | 22702 | 03 |
| 10 | 1.266 | 31.67 | 204345 | 01 |
| TOTAL | 100. | | 16137430 | |

Appendix D.

ANOVA results and summary statistics:

Effect of media on the length of conidia:

1. *A. placenta*-5164

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 3 | 92.186 | 30.729 | 29.35 | <.001*** |
| Residual | 309 | 323.516 | 1.047 | | |
| Total | 312 | 415.702 | | | |

***** Tables of means (adjusted for covariate) *****

Variate: Length

Covariate: Width

Grand mean 12.917

| Media | M102 | MEA | PDA | SDA |
|---|---|---|---|---|
| | 11.893 | 14.644 | 11.993 | 12.889 |
| rep. | 66 | 78 | 71 | 54 |

*** Standard errors of means ***

| Table | Media | |
|---|---|---|
| rep. | unequal | |
| d.f. | 264 | |
| e.s.e. | 0.1700 | min.rep |
| | 0.1414 | max.rep |

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 264 |
| l.s.d. | 0.4733X min.rep |
| | 0.4354 max-min |
| | 0.3938X max.rep |

2. *A. tubulata*-5877

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 3 | 159.5592 | 53.1864 | 56.66 | <.001*** |
| Residual | 353 | 331.3606 | 0.9387 | | |
| Total | 356 | 490.9198 | | | |

***** Tables of means *****

Variate: Length

Grand mean 8.745

| Media | M102 | MEA | PDA | SDA |
|---|---|---|---|---|
| | 9.127 | 9.140 | 7.593 | 9.130 |
| rep. | 80 | 100 | 90 | 87 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 353 |

e.s.e. 0.1083 min.rep

0.0969 max.rep

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 353 |
| l.s.d. | 0.3013X min.rep |
| | 0.2858 max-min |
| | 0.2695X max.rep |

3. *Aschersonia tubulata* 5996

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 2 | 38.885 | 19.442 | 18.95 | <.001*** |
| Residual | 183 | 187.799 | 1.026 | | |
| Total | 185 | 226.684 | | | |

***** Tables of means *****

Variate: Length

Grand mean 9.255

| Media | M102 | MEA | PDA |
|---|---|---|---|
| | 9.917 | 8.917 | 8.962 |
| rep. | 60 | 63 | 63 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 183 |
| e.s.e. | 0.1308 min.rep |
| | 0.1276 max.rep |

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 183 |
| l.s.d. | 0.3649X min.rep |
| | 0.3605 max-min |
| | 0.3561 max.rep |

4. *A. hypocreoidea*-3162

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 3 | 127.0417 | 42.3472 | 47.64 | <.001*** |
| Residual | 276 | 245.3444 | 0.8889 | | |
| Total | 279 | 372.3861 | | | |

***** Tables of means *****

Variate: Length

Grand mean 9.418

| Media | M102 | MEA | PDA | SDA |
|---|---|---|---|---|
| | 9.784 | 10.286 | 8.489 | 9.001 |
| rep. | 75 | 69 | 60 | 76 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 276 |
| e.s.e. | 0.1217 min.rep |
| | 0.1082 max.rep |

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 276 |
| l.s.d. | 0.3389X min.rep |
| | 0.3205 max-min |
| | 0.3011X max.rep |

5. *Aschersonia hypocreoidea* 5269

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 2 | 241.757 | 120.879 | 110.86 | <.001*** |
| Residual | 185 | 201.727 | 1.090 | | |
| Total | 187 | 443.485 | | | |

***** Tables of means *****

Variate: Length

Grand mean 15.348

| Media | M102 | MEA | PDA |
|---|---|---|---|
| | 15.289 | 16.656 | 13.968 |
| rep. | 54 | 70 | 64 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 185 |
| e.s.e. | 0.1421 min.rep |
| | 0.1248 max.rep |

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 185 |
| l.s.d. | 0.3965X min.rep |
| | 0.3731 max-min |
| | 0.3482X max.rep |

Effect of media on the width of conidia:

1. *Aschersonia placenta* 5164

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 3 | 7.84656 | 2.61552 | 27.90 | <.001*** |
| Residual | 265 | 24.83832 | 0.09373 | | |
| Total | 268 | 32.68488 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.854

| Media | M102 | MEA | PDA | SDA |
|---|---|---|---|---|
| | 1.769 | 2.121 | 1.732 | 1.734 |
| rep. | 66 | 78 | 71 | 54 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 265 |
| e.s.e. | 0.0417 min.rep |
| | 0.0347 max.rep |

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 265 |
| l.s.d. | 0.1160X min.rep |
| | 0.1067 max-min |
| | 0.0965X max.rep |

2. *Aschersonia tubulata* 5877

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 3 | 17.82408 | 5.94136 | 133.20 | <.001*** |
| Residual | 353 | 15.74583 | 0.04461 | | |
| Total | 356 | 33.56991 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.493

| Media | M102 | MEA | PDA | SDA |
|---|---|---|---|---|
| | 1.726 | 1.519 | 1.129 | 1.623 |
| rep. | 80 | 100 | 90 | 87 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 353 |
| e.s.e. | 0.0236 min.rep |
| max.rep | 0.211 |

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 353 |
| l.s.d. | 0.0657X min.rep |

0.0623 max-min

0.0587X max.rep

3. *Aschersonia tubulata* 5996

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 2 | 0.25915 | 0.12958 | 2.49 | 0.086 |
| Residual | 183 | 9.51487 | 0.05199 | | |
| Total | 185 | 9.77402 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.591

| Media | M102 | MEA | PDA |
|---|---|---|---|
| | 1.586 | 1.549 | 1.639 |
| rep. | 60 | 63 | 63 |

*** Standard errors of means ***

Table Media

rep. unequal

d.f. 183

e.s.e. 0.0294 min.rep

0.0287 max.rep

*** Least significant differences of means ***

Table Media

rep. unequal

d.f. 183

l.s.d. 0.0821X min.rep

0.0812 max-min

0.0802 max.rep

4. *Aschersonia hypocreoidea* 3162

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 3 | 2.5886 | 0.8629 | 4.62 | 0.004 |
| Residual | 276 | 51.5937 | 0.1869 | | |
| Total | 279 | 54.1822 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.549

| Media | M102 | MEA | PDA | SDA |
|---|---|---|---|---|
| | 1.587 | 1.471 | 1.430 | 1.675 |
| rep. | 75 | 69 | 60 | 76 |

*** Standard errors of differences of means ***

Table Media

rep. unequal

d.f. 276

s.e.d. 0.0789X min.rep

0.0747 max-min

0.0701X max.rep

*** Least significant differences of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 276 |
| l.s.d. | 0.1554X min.rep |
| | 0.1470 max-min |
| | 0.1381X max.rep |

5. *Aschersonia hypocreoidea* 5269

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Media | 2 | 0.43950 | 0.21975 | 3.15 | 0.045 |
| Residual | 185 | 12.88727 | 0.06966 | | |
| Total | 187 | 13.32678 | | | |

******* Tables of means *****

Variate: Width

Grand mean 2.179

| Media | M102 | MEA | PDA |
|---|---|---|---|
| | 2.187 | 2.230 | 2.116 |
| rep. | 54 | 70 | 64 |

*** Standard errors of means ***

| Table | Media |
|---|---|
| rep. | unequal |
| d.f. | 185 |

e.s.e. 0.0359 min.rep

0.0315 max.rep

*** Least significant differences of means ***

Table Media

rep. unequal

d.f. 185

l.s.d. 0.1002X min.rep

0.0943 max-min

0.0880X max.rep

Effect of temperature on the length of conidia:

1. *Aschersonia placenta* 5164

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 5 | 96.059 | 19.212 | 18.84 | <.001*** |
| Residual | 447 | 455.912 | 1.020 | | |
| Total | 452 | 551.971 | | | |

***** Tables of means *****

Variate: Length

Grand mean 11.876

| Temp | 15.00 | 20.00 | 22.00 | 25.00 | 28.00 | 30.00 |
|---|---|---|---|---|---|---|
| | 11.054 | 11.821 | 11.883 | 12.451 | 12.229 | 11.681 |
| rep. | 77 | 70 | 71 | 100 | 65 | 70 |

*** Standard errors of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 447 |
| e.s.e. | 0.1253 min.rep |
| | 0.1010 max.rep |

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 447 |
| l.s.d. | 0.3482X min.rep |
| | 0.3162 max-min |
| | 0.2807X max.rep |

2. *Aschersonia tubulata* 5877

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 4 | 194.0236 | 48.5059 | 49.13 | <.001*** |
| Residual | 362 | 357.4257 | 0.9874 | | |
| Total | 366 | 551.4493 | | | |

***** Tables of means *****

Variate: Length

Grand mean 8.700

| Temp | 20.00 | 22.00 | 25.00 | 28.00 | 30.00 |
|---|---|---|---|---|---|
| | 7.595 | 8.962 | 9.283 | 9.489 | 8.343 |
| rep. | 90 | 63 | 84 | 70 | 60 |

*** Standard errors of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 362 |
| e.s.e. | 0.1283 min.rep |
| | 0.1047 max.rep |

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 362 |
| l.s.d. | 0.3568X min.rep |
| | 0.3257 max-min |
| | 0.2913X max.rep |

3. *Aschersonia tubulata* 5996

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 4 | 243.574 | 60.894 | 54.37 | <.001 *** |
| Residual | 388 | 434.533 | 1.120 | | |
| Total | 392 | 678.107 | | | |

***** Tables of means *****

Variate: Length

Grand mean 9.111

| Temp | 15.00 | 20.00 | 22.00 | 25.00 | 28.00 |
|---|---|---|---|---|---|
| | 8.108 | 8.880 | 8.962 | 10.115 | 10.153 |
| rep. | 100 | 100 | 63 | 65 | 65 |

*** Standard errors of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 388 |
| e.s.e. | 0.1333 min.rep |
| | 0.1058 max.rep |

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 388 |
| l.s.d. | 0.3707X min.rep |
| | 0.3347 max-min |
| | 0.2942 max.rep |

4. *Aschersonia hypocreoidea* 3162

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | Fpr. |
|---|---|---|---|---|---|
| Temp | 3 | 62.7688 | 20.9229 | 23.53 | <.001 *** |

| | | | |
|---|---|---|---|
| Residual | 244 | 216.9229 | 0.8890 |
| Total | 247 | 279.6917 | |

***** Tables of means *****

Variate: Length

Grand mean 9.052

| Temp | 20.00 | 22.00 | 25.00 | 28.00 |
|---|---|---|---|---|
| | 8.885 | 8.490 | 8.935 | 9.855 |
| rep. | 64 | 60 | 60 | 64 |

*** Standard errors of means ***

Table Temp

rep. unequal

d.f. 244

e.s.e. 0.1217 min.rep

0.1179 max.rep

*** Least significant differences of means ***

Table Temp

rep. unequal

d.f. 244

l.s.d. 0.3391 min.rep

0.3337 max-min

0.3283 max.rep

5. *Aschersonia hypocreoidea* 5269

***** Analysis of variance *****

Variate: Length

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 2 | 59.680 | 29.840 | 22.08 | <.001 *** |
| Residual | 192 | 259.422 | 1.351 | | |
| Total | 194 | 319.102 | | | |

***** Tables of means *****

Variate: Length

Grand mean 13.551

| Temp | 15.00 | 20.00 | 22.00 |
|---|---|---|---|
| | 12.769 | 13.916 | 13.968 |
| rep. | 65 | 66 | 64 |

*** Standard errors of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 192 |
| e.s.e. | 0.1453 min.rep |
| | 0.1431 max.rep |

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 192 |
| l.s.d. | 0.4053X min.rep |
| | 0.4022 max-min |
| | 0.3991X max.rep |

Effect of temperature on the width of conidia:

1. *Aschersonia placenta* 5164

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 5 | 4.0079 | 0.8016 | 2.77 | 0.018* |
| Residual | 447 | 129.2010 | 0.2890 | | |
| Total | 452 | 133.2089 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.783

| Temp | 15.00 | 20.00 | 22.00 | 25.00 | 28.00 | 30.00 |
|---|---|---|---|---|---|---|
| | 1.728 | 1.786 | 1.732 | 1.906 | 1.871 | 1.633 |
| rep. | 77 | 70 | 71 | 100 | 65 | 70 |

*** Standard errors of means ***

| Table | Temp | |
|---|---|---|
| rep. | unequal | |
| d.f. | 447 | |
| e.s.e. | 0.0667 | min.rep |
| | 0.0538 | max.rep |

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 447 |

l.s.d. 0.1853X min.rep

0.1683 max-min

0.1494X max.rep

2. *Aschersonia tubulata* 5887

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 4 | 19.47448 | 4.86862 | 82.07 | <.001 *** |
| Residual | 362 | 21.47505 | 0.05932 | | |
| Total | 366 | 40.94953 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.447

| Temp | 20.00 | 22.00 | 25.00 | 28.00 | 30.00 |
|---|---|---|---|---|---|
| | 1.129 | 1.638 | 1.570 | 1.288 | 1.737 |
| rep. | 90 | 63 | 84 | 70 | 60 |

*** Standard errors of means ***

Table Temp

rep. unequal

d.f. 362

e.s.e. 0.0314 min.rep

0.0257 max.rep

*** Least significant differences of means ***

| | |
|---|---|
| Table | Temp |
| rep. | unequal |
| d.f. | 362 |
| l.s.d. | 0.0874X min.rep |
| | 0.0798 max-min |
| | 0.0714X max.rep |

3. *Aschersonia tubulata* 5996

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 4 | 0.94620 | 0.23655 | 5.83 | <.001 *** |
| Residual | 388 | 15.75080 | 0.04059 | | |
| Total | 392 | 16.69699 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.549

| Temp | 15.00 | 20.00 | 22.00 | 25.00 | 28.00 |
|---|---|---|---|---|---|
| | 1.548 | 1.483 | 1.638 | 1.562 | 1.553 |
| rep. | 100 | 100 | 63 | 65 | 65 |

*** Standard errors of means ***

| | |
|---|---|
| Table | Temp |
| rep. | unequal |
| d.f. | 388 |
| e.s.e. | 0.0254 min.rep |

0.0201 max.rep

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 388 |
| l.s.d. | 0.0706X min.rep |
| | 0.0637 max-min |
| | 0.0560 max.rep |

4. *Aschersonia hypocreoidea* 3162

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 3 | 2.53362 | 0.84454 | 16.57 | <.001 *** |
| Residual | 244 | 12.43337 | 0.05096 | | |
| Total | 247 | 14.96699 | | | |

***** Tables of means *****

Variate: Width

Grand mean 1.598

| Temp | 20.00 | 22.00 | 25.00 | 28.00 |
|---|---|---|---|---|
| | 1.610 | 1.431 | 1.635 | 1.707 |
| rep. | 64 | 60 | 60 | 64 |

*** Standard errors of means ***

| Table | Temp |
|---|---|
| rep. | unequal |

d.f. 244

e.s.e. 0.0291 min.rep

0.0282 max.rep

*** Least significant differences of means ***

Table Temp

rep. unequal

d.f. 244

l.s.d. 0.0812 min.rep

0.0799 max-min

0.0786 max.rep

5. *Aschersonia hypocreoidea* 5269

***** Analysis of variance *****

Variate: Width

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Temp | 2 | 0.09285 | 0.04642 | 0.56 | 0.570 |
| Residual | 192 | 15.80483 | 0.08232 | | |
| Total | 194 | 15.89767 | | | |

***** Tables of means *****

Variate: Width

Grand mean 2.091

| Temp | 15.00 | 20.00 | 22.00 |
|---|---|---|---|
| | 2.062 | 2.096 | 2.115 |
| rep. | 65 | 66 | 64 |

*** Standard errors of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 192 |
| e.s.e. | 0.0359 min.rep |
| | 0.0353 max.rep |

*** Least significant differences of means ***

| Table | Temp |
|---|---|
| rep. | unequal |
| d.f. | 192 |
| l.s.d. | 0.1000X min.rep |
| | 0.0993 max-min |
| | 0.0985X max.rep |