Diss. ETH No. 13869

Cultivation, sporulation and phylogenetic analysis of *Neozygites parvispora* and *Entomophthora thripidum*, two fungal pathogens of thrips

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
DOCTOR OF NATURAL SCIENCES

presented by

Florian Matthias FREIMOSER
Dipl. Natw. ETH Zurich
Born March 10th, 1973
Citizen of Germany

Accepted on the recommendation of

Prof. Dr. Markus Aebi, examiner
Prof. Dr. Jørgen Eilenberg, coexaminer
Dr. Urs Tuor, coexaminer

2000

Acknowledgements

The journey of my PhD study is coming to the end and I am struggling with the last and most fascinating and challenging steps on this path. In retrospect the strenuous, boring or disappointing experiences become insignificant and only make the beautiful, joyful, successful and lucky moments more rewarding. In this sense I am thankful to every person who joined or crossed my path during this time and made it an instructive experience or even an exciting adventure. Without diminishing the value of the scientific apprenticeship it is this observation of or interaction and collaboration with other people that was the most important and educational lesson during my PhD.

I would especially like to thank Prof. Markus Aebi, for the possibility to do this PhD under his supervision, for the interest in my work, and his trust in my plans and decisions. Above all I enjoyed the direct and open discussions and scrupulous decisions; even when we had differing opinions. And his fascination and excitement is contagious and reassuring.

I also thank Dr. Urs Tuor for his courage and persistence in starting and leading the "mycoinsecticide project". I especially appreciate his openness and willingness to debate problems. The possibility to work in his group was always interesting and fascinating and helped me to gain invaluable experience.

I would also like to thank past and present members of the mycoinsecticide, yeast and coprinus group who contributed to the success and my happiness during this work. Similarly, all members of the "Institute of Microbiology" that created a friendly and comfortable working atmosphere are acknowledged. Especially I would like to thank Anne because we walked together on this PhD track, for the critical but also assuring comments on my work and the joyful adventures outside of work.

I am also grateful to Prof. Jørgen Eilenberg for his agreement to serve as a co-examiner of this thesis.

Finally I would like to express my appreciation to Prof. Nikolaus Amrhein who encouraged me to ask for and start my PhD studies in the group of Prof. Markus Aebi. I am in particular thankful for his interest and advise for my plans and the discussions since the time when I started to study at ETH.

My unlimited thank belongs to my mother and father and all people who encouraged me through words and action to follow my interests.

CONTENT

Summary 1

Zusammenfassung 3

CHAPTER 1 5

Introduction

1.1 Morphologic characteristics that are important for the classification of Entomophthorales **8**

1.1.1 Nuclear characteristics are important for the distinction at the family level 9

1.1.2 Primary sporophores and spores are important for the separation of entomophthoralean genera 10

1.1.2.1 Primary sporophores *11*

1.1.2.2 Primary spores *11*

1.1.2.3 Mechanism of spore discharge *12*

1.1.2.4 Secondary spores *13*

1.1.2.5 Resting spores *13*

1.1.2.6 Rhizoids and pseudocystidia *13*

1.1.3 Quantitative morphological characteristics are used to distinguish different species 15

1.2 From obligate biotrophic pathogens to mycoinsecticides? The *in vivo* and *in vitro* life cycle of Entomophthorales **17**

1.2.1 The *in vivo* life cycle of Entomophthorales 17

1.2.2 *In vitro* isolation, cultivation and sporulation of entomophthoralean pathogens 19

1.2.2.1 Isolation *19*

1.2.2.2 Cultivation *20*

1.2.2.3 Sporulation *21*

1.2.2.4 Field experiences with entomophthoralean pathogens *22*

CHAPTER 2 29

Phylogenetic analysis of SSU rDNA sequences suggests a common origin of Neozygitaceae (Zygomycota; Entomophthorales), fungal pathogens of arthropods, and of microsporidia, obligate intracellular parasites

CHAPTER 3 45

The MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities

CHAPTER 4 53

Characterization of growth factors from hemolymph for the cultivation of the entomopathogenic fungus *Neozygites parvispora*

CHAPTER 5 71

In vitro cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements and sporulation

CHAPTER 6 87

Regulation of hyphal growth and sporulation of the insect pathogenic fungus *Entomophthora thripidum in vitro*

CHAPTER 7 109

Conclusions, opinions and outlook

Summary

This thesis describes studies with the two fungi *Neozygites parvispora* and *Entomophthora thripidum* that are both pathogenic to *Thrips tabaci* (onion or potato thrips; Thysanoptera). *N. parvispora* has been isolated previously for the first time in this laboratory and the accomplishment of the *in vitro* cultivation of *E. thripidum* is one of the results of this work.

To investigate the evolutionary relationship of Neozygitaceae within the Entomophthorales the small subunit ribosomal DNA (ssu rDNA) sequences of several species were determined and analyzed phylogenetically. For this analysis the sequences of microsporidia were included because these important intracellular parasites of insects and other animals have been related to Entomophthorales recently. This study confirmed at the molecular level the monophyletic origin of Neozygitaceae and their belonging to the Entomophthorales as well as the fungal origin of microsporidia. Most interestingly it was revealed that microsporidia originated from within the Entomophthorales and formed a sister group to Neozygitaceae. It was therefore concluded that microsporidia have their origin within the phylum of the Zygomycota, belong to the Entomophthorales and are most closely related to Neozygitaceae. This represented the first localization of the origin of microsporidia within the fungal kingdom.

The main body of this PhD thesis consisted of the isolation of a growth factor from hemolymph that is essential for growth of *N. parvispora* under laboratory conditions. For this reason the MTT assay was first developed for the rapid colorimetric determination of fungal cell densities in small culture volumes. Fractionation of hemolymph revealed the presence of a HMW (high molecular weight) and a LMW (low molecular weight) fraction that both had growth promoting activity. Since the HMW acitivity could be replaced by adding FBS (fetal bovine serum) we focused on the purification of the LMW activity, which was specific for hemolymph and could not be replaced. The LMW growth promoting activity resisted heating to 100°C and digestion with peptidases, had a molecular weight between 100 and 500 Da and was inactivated by acid treatment. Further purification was performed on Sephadex and Dowex resins, but did not result in sufficient material for the structural identification of possible growth factors. This study was concluded with the assertion that much more hemolymph would be required for the successful purification of growth promoting substances.

The second fungal species studied during this PhD was *E. thripidum*. We isolated this fungus for the first time from infected but still living thrips in a

complex liquid medium. *E. thripidum* is noteworthy for its vegetative growth in the form of protoplasts that could be continuously cultured in Grace's insect cell culture medium or in GLEN medium that were both supplemented with FBS. The protoplasts aggregated *in vitro* thus forming dense pellets. If these protoplast pellets were kept in the same culture medium for 10 to 20 days they underwent a differentiation process that led to cell wall formation, hyphal growth and mycelium. This switch from protoplasts to mycelium was a prerequisite for the production of infectious spores, which took place after the transfer onto water agar. These spores were infectious to the original host and the fungus could be reisolated from such infected thrips. A more detailed investigation of the differentiation process revealed that *E. thripidum* produced and secreted a factor that autoinduced the formation of mycelium. The differentiation to mycelium was inhibited by nitrogen. Furthermore it was found that instead of mycelium some isolates formed huge spherical cells that led to structures similar to resting spores. In analogy to the induction of mycelium resting spores were autoinduced.

Zusammenfassung

Während dieser Doktorarbeit habe ich die zwei Pilze *Neozygites parvispora* und *Entomophthora thripidum* studiert, welche beide natürliche Pathogene von *Thrips tabaci* (Kartoffel- oder Zwiebelthrips; Fransenflügler; Thysanoptera) sind. Beide Pilze wurden in unserem Labor zum ersten Mal isoliert und kultiviert. Das Erreichen der *in vitro* Kultivierung von *E. thripidum* ist eines der Resultate dieser Arbeit.

Um die phylogenetische Beziehung von *Neozygites* zu den übrigen Entomophthorales zu untersuchen wurden SSU rDNA (DNS der kleinen Ribosomen-Untereinheit) Sequenzen von mehreren Arten bestimmt und phylogenetisch analysiert. In diese Analyse wurden auch Sequenzen von Mikrosporidien eingeschlossen, da diese bedeutenden intrazellulär lebenden Parasiten von Insekten und anderen Tieren vor kurzem mit Entomophthorales in Verbindung gebracht wurden. Dabei wurden der monophyletische Ursprung der Neozygitaceae und deren Zugehörigkeit zu den Entomophthorales wie auch die Zugehörigkeit der Mikrosporidien zum Reich der Pilze auf molekularer Ebene bestätigt. Interessanterweise hatten die Mikrosporidien ihren Ursprung innerhalb der Entomophthorales und bildeten eine Tochtergruppe zu den Neozygitaceae. Daraus wurde geschlossen, dass der Ursprung der Mikrosporidien innerhalb des Phylums Zygomycota liegt, dass Mikrosporidien zu den Entomophthorales gehören und innerhalb dieser am nächsten verwandt zu den Neozygitaceae sind. Dies stellte die erste Lokalisierung des Ursprungs von Mikrosporidien innerhalb des Reiches der Pilze dar.

Die Hauptarbeit dieser Doktorarbeit bestand in der Isolation von Wachstumsfaktoren aus Hämolymphe, welche für die Kultivierung von *N. parvispora* unerlässlich ist. Hierfür wurde zuerst der MTT-Test entwickelt, der die schnelle kolorimetrische Bestimmung der Zelldichte in kleinen Kulturvolumina ermöglicht. Die Insektenhämolymphe wurde in eine hochmolekulare und eine niedermolekulare wachstumsfördernde Fraktion aufgetrennt. Da die hochmolekulare Aktivität mit FBS (fötales Rinderserum) ersetzt werden konnte, haben wir uns auf die Reinigung der niedermolekularen Wachstumsaktivität konzentriert. Diese Fraktion war spezifisch für Insektenhämolymphe und konnte nicht ersetzt werden. Es wurde gefunden, dass die niedermolekulare Aktivität resistent gegenüber Erhitzen auf 100°C und Abbau durch Peptidasen war, dass das Molekulargewicht zwischen 100 und 500 Da lag, und dass diese Aktivität nach Säurebehandlung nicht mehr vorhanden war. Bei der weiteren Auftrennung auf Sephadex und Dowex Säulenmaterialien konnte nicht genügend Material zurückgewonnen werden,

um mögliche Wachstumsfaktoren strukturell zu identifizieren. Diese Studie schliesst mit der Aussage, dass wesentlich grössere Mengen Insektenhämolymphe nötig sind, um die wachstumsfördernden Substanzen erfolgreich isolieren zu können.

Der zweite Pilz, welcher während diesem Doktorat studiert wurde, ist *E. thripidum*. Wir haben diesen Pilz von infizierten, aber noch lebenden Thripsen in einem komplexen Flüssigmedium isoliert. *E. thripidum* ist wegen des vegetativen Wachstums in der Form von langgestreckten Protoplasten bemerkenswert. Diese Protoplasten konnten in Grace's Insektenzellkulturmedium oder in GLEN Medium nach der Zugabe von FBS kontinuierlich gezüchtet werden. *In vitro* aggregierten die einzelnen Protoplastenzellen und bildeten dichte Zellhaufen. Wenn diese Protoplasten-Haufen während 10 bis 20 Tagen im selben Medium belassen wurden durchlief der Pilz einen Differentiationsprozess, der zu Zellwandbildung, Hyphenwachstum und Myzelbildung führte. Dieser Wechsel vom Protoplasten- in das Myzel-Stadium war Voraussetzung für die Ausbildung von infektiösen Sporen, welche nach dem Transfer des Myzels auf Wasseragar gebildet wurden. Die *in vitro* produzierten Sporen konnten benutzt werden, um das ursprüngliche Wirtsinsekt zu infizieren. Daraufhin konnte der Pilz wieder aus diesen künstlich infizierten Thripsen isoliert werden. Detaillierte Studien zum Differenzierungsprozess zeigten, dass *E. thripidum* einen Faktor produzierte und sekretierte, der die Myzelbildung induzierte. Diese Differenzierung wurde durch die Anwesenheit von Stickstoffquellen verhindert. Es wurde des weiteren gefunden, dass manche Isolate von *E. thripidum* anstelle von Myzel grosse runde Zellen bildeten. Diese Kugeln entwickelten sich schliesslich zu Strukturen, die den Dauersporen der Entomophthorales sehr ähnlich sahen. Analog zur Myzelbildung war auch diese Dauersporenbildung selbstinduziert.

CHAPTER 1

INTRODUCTION

Introduction

Whenever plants are cultivated or animals are bred new environments for other organisms that feed on these cultivated plants and animals are created. Furthermore, commerce between continents and the deliberate introduction of new crops, ornamentals and animals from foreign continents brought along the introduction of pests which then often thrived in their new habitats (Sailer, 1983; for a comprehensive account see also Crosby, 1996). Therefore, agriculture and animal husbandry usually make the control of such pest organisms necessary. For this, the idea to control undesired animals, plants or microorganisms biologically by using other antagonistic organisms is opposed to the method of killing pest organisms with chemical pesticides. The latter approach has been commonly used to solve the pest problems that appeared due to intensified agriculture and newly introduced plagues. However, it is now realized that the „pesticide boom" not only reduced many pest problems (at least temporarily), but also caused the destruction of beneficial organisms or pesticide poisonings in humans (Debach & Rosen, 1991).

The use of natural enemies to control pest populations and the first observations and descriptions of microbial diseases of insects date back more than 2000 years. At this time Chinese already used predatory ants to control pest insects in their citrus gardens (Coulson *et al.*, 1982; cited in Debach & Rosen, 1991). Insects that were parasitized and thus mummified by fungal pathogens symbolized "perennial youth and immortality" and were also used as a drug in China (Hoffmann, 1947; Kobayasi, 1977). The disease of the silkworm, also known as muscardine disease, was supposedly observed first in the Orient as sericulture became one of the major industries (Steinhaus, 1956). However, it was not until 1834 when Agostino Bassi showed for the first time that a microorganism, the fungus *Beauveria bassiana*, is the cause of the disease (Ainsworth, 1956). Bassi thus not only disproved the concept of spontaneous creation, but also introduced the concept of pathogenicity or the germ theory of diseases. Bassi's work is also considered as the fountain for the idea of microbial biocontrol (Steinhaus, 1956). However, concrete and specific suggestions for the use of insect pathogenic fungi and other microorganisms as pest control agents, were made only three decades later almost simultaneously in the United States by John Lawrence LeConte and in central and eastern Europe by Louis Pasteur and Elie Metchnikoff and others (Steinhaus, 1956). Metchnikoff, who discovered the fungus now called *Metarhizium anisopliae* succeeded in mass producing entomopathogenic microorganisms *in vitro* and

proposed to use them to infect pest organisms in the field (Steinhaus, 1956). Historically entomopathogenic fungi were thus important for the development of concepts like pathogenicity or disease, and also their practical value for the control of insect pests was envisioned already long ago.

The order of the Entomophthorales, which consists almost exclusively of obligate "entomotrophic" or "acaritrophic" fungi, is known for more than 100 years. The first acknowledged description of an entomophthoralean fungus has been made by Cohn (1855a, b), who described *Entomophthora muscae.* This fungus attracted the interest of early naturalists, because it causes the impressive disease that makes houseflies stick to windowpanes or walls surrounded by a characteristic halo of ejected spores. This striking sickness of *Musca domestica* had already been observed earlier and it inspired Goethe to the description of a "destroying dusting" of the housefly (1820). Ever since Entomophthorales attracted the interest of mycologists and entomologists alike and the meticulous and thorough descriptions of early naturalists like Brefeld (1877), Thaxter (1888) or Schweizer (1948) are probably still the most fascinating and enjoyable introductions for anybody interested in this group of insect pathogenic fungi.

1.1 MORPHOLOGIC CHARACTERISTICS THAT ARE IMPORTANT FOR THE CLASSIFICATION OF ENTOMOPHTHORALES

Already with the first description of an entomophthoralean fungus, the pathogen now called *Entomophthora muscae*, the foundation for later confusions and disputes was laid. This species was originally described under the name of *Empusa muscae* (Cohn, 1855a, b). Fresenius (1856) criticized correctly, that *Empusa* was already used for a genus in orchids, and suggested the name *Entomophthora* instead. Some authors followed his suggestion and adopted the name *Entomophthora*, while others kept using *Empusa* or used an even different designation. Again others used both names interchangeably or separated two genera of Entomophthoraceae (reviewed in: Hall & Bell, 1962; Remaudière & Hennebert, 1980; Remaudière & Keller, 1980). Meanwhile the Entomophthorales have been separated into at least three families containing arthropod pathogenic fungi and, depending on the author, 12 to 14 genera are distinguished (Tab. 1).

In the first description entomophthoralean fungi were placed to the Acmosporiacei (=Aspergillaceae) (Cohn, 1855). Other authors associated them with Oomycota, as a stage in the life cycle of *Saprolegnia*, or with *Saccharomyces* (summarized in Thaxter, 1888). Brefeld arranged them in the

Basidiomycota (1877), while Thaxter also used the term "basidium" to designate the sporophore but correctly classified Entomophthorales in the Zygomycota (1888). This phylum is mainly characterized by the formation of nonmotile sporangiospores (Cavalier-Smith, 1987), but the presence of sexual zygospores and of chitin and chitosan in their cell walls have been considered as important characteristics as well (Bartnicki-Garcia, 1968; Hesseltine & Ellis, 1973). According to Cavalier-Smith (1987) zygospores are also present in Chytridiomycota, even though these structures might be called differently in this class. The same author does not consider Chytridiomycota and Zygomycota sufficiently distinct to merit two separate phyla (Cavalier-Smith, 1987; Cavalier-Smith, 1998). Entomophthorales differ from the general characters of Zygomycota by the presence of glucan and the absence of chitosan in the cell wall (Hoddinott & Olsen, 1972). The sporangium of Entomophthorales is reduced to a single spored sporangium, and this spore is actively discharged (Thaxter, 1888; Hesseltine & Ellis, 1973). As a consequence, spores of entomophthoralean fungi have two cell wall layers, one originating from the sporangiospore and the other from the sporangium.

1.1.1 NUCLEAR CHARACTERISTICS ARE IMPORTANT FOR THE DISTINCTION AT THE FAMILY LEVEL

Originally Entomophthorales consisted only of the familiy Entomophthoraceae, but now they have been divided into as much as six different families (Humber, 1989). Of these, only the members of three families commonly appear as insect or mite pathogens: the Ancylistaceae, the Entomophthoraceae and the Neozygitaceae. These three families are mainly distinguished based on the morphology of their nuclei when stained with various dyes. An important criterion with respect to nuclear morphology is the presence or absence of condensed chromatin that strongly stains with aceto-carmine, aceto-orcein, bismarck brown Y or lactophenol cotton blue (defined in Ben-Ze'ev *et al.*, 1987). The nuclear features of the three families are summarized according to descriptions of Ben-Ze'ev and Kenneth (1982), Ben-Ze'ev *et al.* (1987) and Humber (1981; 1989):

- *Ancylistaceae*: Nuclei small (3-5 µm in diameter during interphase) and difficult to observe during mitosis, 1-2 prominent central nucleoli that are persistent and stain stronger during mitosis, without condensed chromatin, nuclear envelope remains intact throughout mitosis, metaphase spindle centrally located and occupying only a small volume of the nucleus.

- *Entomophthoraceae*: Nuclei typically 5-12 µm in diameter (but sometimes smaller) and visible during mitosis, 1-2 inconspicuous central nucleoli that remain intact during mitosis, striking condensed chromatin, nuclear envelope remains intact during mitosis, metaphase spindle eccentrically located and occupying a small part of nuclear volume.
- *Neozygitaceae*: Nuclei small (3-5 µm during interphase), nuclei staining moderately with aceto-carmine or aceto-orcein and poorly or not at all with bismarck brown Y (= "semi-condensed" chromatin; Ben-Ze'ev *et al.*, 1987), 1 ovoid nucleolus, nuclear envelope remains intact during mitosis, synchronous mitoses of all nuclei of a cell, mitotic spindle centrally located and occupying most of the nuclear volume.

The Basidiobolaceae comprise a fourth family that is said to belong to the Entomophthorales. This group is distinguished by large nuclei (>10 µm), a nucleolus that disperses during mitosis, the absence of condensed chromatin and a nuclear envelope that breaks down during mitosis. Furthermore, Basidiobolaceae exhibit a special "rocket-like" spore discharge mechanism (Ingold, 1934). However, the members of this family are not commonly pathogenic to insects and rather occur as saprobes and unspecific colonizers and pathogens of animals including man (Humber, 1989; Gugnani, 1999). Based on phylogenetic analysis of ribosomal DNA sequences the belonging of the Basidiobolaceae to the Entomophthorales has been questioned and a close association with Chytridiomycetes was found (Nagahama *et al.*, 1995; Jensen *et al.*, 1998). The family of the Basidiobolaceae will not be considered during the remainder of this discussion.

The Neozygitaceae were the last to be defined as a separate family (Ben-Ze'ev *et al.*, 1987), which prompted Humber (1989) to reevaluate family and generic characteristics that are of taxonomic value. This led to the creation of several new families and genera and to a rearrangement of existing taxa (Humber, 1989). This revised taxonomy serves as guideline for the following discussion.

1.1.2 PRIMARY SPOROPHORES AND SPORES ARE IMPORTANT FOR THE SEPARATION OF ENTOMOPHTHORALEAN GENERA

The Ancylistaceae and the Neozygitaceae each contain only one genus, but within the Entomophthoraceae a dozen or more separate genera are recognized. However, this classification is still being discussed and there is no agreement upon which characters are acceptable as taxonomic criteria at the different taxonomic levels. In the following an attempt is made to give an

overview of the morphological characteristics of Entomophthorales used for their classification. The discussion will closely follow the view of entomophthoralean genera as defended by Humber (1981; 1989), Ben-Ze'ev and Kenneth (1982), Ben-Ze'ev *et al.* (1987), Balazy (1993) and Remaudière and Keller (1980). The most important characters that are considered for the classification of entomophthoralean genera are:

- Characteristics of the primary sporophores
- Shape, nuclear number and wall structure of primary spores
- Mechanism of primary spore discharge
- Types and characteristics of secondary spores
- Characteristics of resting spores
- Presence and morphology of rhizoids and pseudocystidia

The different genera that are distinguished with these characters are listed in Table 1. More detailed descriptions of different entomophthoralean genera and discussions of taxonomic issues concerning this group of fungi are found in the above mentioned articles as well as in these reports: Gustafsson, 1965; MacLeod *et al.*, 1976; Remaudière & Hennebert, 1980; Wilding & Brady, 1984a-d; Keller, 1987; Humber & Feng, 1991; Keller, 1991; Balazy, 1993; Keller, 1993; Keller & Eilenberg, 1993; Keller, 1997; Steinkraus *et al.*, 1998; Keller *et al.*, 1999.

1.1.2.1 Primary sporophores

Sporophores of entomophthoralean fungi are described as either simple or branched. A simple sporophore can also be branched, but is not divided by septa and therefore remains as a unicellular coenocytic structure. Branched sporophores are septated so that there is usually one nucleus in each spore-forming cell. The branched type of sporophores is associated with uninucleate, bitunicate primary spores, while simple sporophores form multinucleate and unitunicate primary spores.

1.1.2.2 Primary spores

The shape of primary conidia ranges from spherical to elongate, ovoid or cylindrical and has been used as a help for species identification by Hutchinson (1963), Gustafsson (1965) and MacLeod *et al.* (1976). Most recently Remaudière and Keller (1980) used spore morphology as a criterion of their classification. However, the shape of primary spores has been rejected as a character to distinguish entomophthoralean genera but is still useful for the

description of different genera (Humber, 1981; Ben-Ze'ev & Kenneth, 1982; Humber, 1989).

Based on the number of nuclei in primary spores genera with uni- or multinucleate spores can be distinguished. The group with more than one nucleus per primary spore can be further divided into genera with usually four or eight nuclei, up to 20 nuclei or with more than 20 nuclei in each primary spore.

Primary spores are also distinguished by the appearance of their cell wall after discharge, which can either be unitunicate or bitunicate. In bitunicate spores two distinct and separate layers are visible after the spore has been discharged. In unitunicate spores only one such cell wall layer is recognizable. This criterion only relates to whether or not two separate layers are visible in discharged spores. It is not meant that Entomophthorales have spores with either one or two cell wall layers. As mentioned before Entomophthorales form single spored sporangia and therefore display one wall layer originating from the sporangiospore and another one from the sporangium. However, the sporangial nature of these spores is not accepted by all authorities and neither is the distinction into which species are bitunicate and which are unitunicate (see for example Eilenberg *et al.*, 1986; Eilenberg *et al.*, 1995).

1.1.2.3 Mechanism of spore discharge

A characteristic feature of Entomophthorales is the active spore discharge mechanism in many species. Spore discharge in a variety of fungi has been studied extensively by Ingold and also the different mechanisms of ejection are summarized in the work of this author (Ingold, 1934; Ingold, 1966). The different types of forcible spore discharge are the following:

- *Rounding off mechanism*: High turgor pressure in the sporophore and/or the spore cause the sudden rounding off of the cell walls between the sporophore and the spore. In most genera both the sporophore and the spore round off, while in *Conidiobolus* only the spore cell wall bulges out (Martin, 1925).
- *Sporophore explosion*: In the genus *Entomophthora* the turgid sporophore explodes thus ejecting the spore together with a drop of cytoplasm of the conidiophore. This mechanism is similar to that of *Pilobolus* (Ingold, 1934; Ingold, 1953).
- *Passive spore liberation*: In the families of the Ancylistaceae, Entomophthoraceae and Neozygitaceae passive spore release only occurs in species forming capillispores and in *Massospora* that probably lost its active spore discharge secondarily (Evans, 1989).

1.1.2.4 Secondary spores

The capability to produce secondary spores, sometimes even of different types, is another remarkable feature of Entomophthorales. There are three clearly distinct forms of secondary spores:

- *Single forcibly ejected secondary spores* are formed directly from the primary spore that germinates and serves as a one-celled sporophore (Ben-Ze'ev & Kenneth, 1982).
- *Microspores* are produced in multiple from a single primary spore. They are forcibly discharged and are similar to the primary spore except for their smaller size.
- *Capillispores* are remarkable structures that form at the top of a long slender capillary tube. The spore is not ejected but "waits" to be detached by insects or mites. The capillispore bears a gluey droplet at the tip, which makes it stick to the cuticle. In entomophthoralean species with capillispores the primary spore serves as the dispersive unit, while the capillispore comprises the infectious particle.

The types of secondary spores formed are again not consistent in groups of otherwise homogenous genera and for this reason they are not valued as taxonomic criteria on the generic level (Humber, 1981; Ben-Ze'ev & Kenneth, 1982).

1.1.2.5 Resting spores

Resting spores are thick walled structures that serve as resting stage when the host is not available or for overwintering. They are separated into sexually formed zygospores and asexually emerging azygospores. However, resting spores have not been observed in all species and knowledge about the nuclear events during resting spore formation is very limited. For these reasons taxonomical value of resting spores is disregarded (Remaudière & Keller, 1980; Humber, 1981).

1.1.2.6 Rhizoids and pseudocystidia

Rhizoids are fungal structures that attach the insect host to the surface. Three types of rhizoids are distinguished (summarized in Humber, 1981; Ben-Ze'ev & Kenneth, 1982): numerous individual, little differentiated rhizoids; numerous rhizoids that aggregate into one or more columnar rhizomorphs; and few thick rhizoids with strongly differentiated terminal holdfasts. The fact that

some species seem not to form rhizoids consistently prompted dispute concerning the taxonomic value of these structures (Remaudière & Keller, 1980). Humber (1981) and others (Brobyn & Wilding, 1977; Ben-Ze'ev & Kenneth, 1982) consider the general absence or the frequent presence of rhizoids in different species as taxonomically important, at least on subgeneric level. Differences in the morphology of the rhizoids prompted Humber (1989) to divide the genus *Erynia* sensu lato (as defined by Remaudière & Hennebert, 1980; Remaudière & Keller, 1980; and Keller 1991) into the genera *Erynia*, *Furia* and *Pandora*. Rhizoids are absent in *Entomophaga*, *Massospora*, *Neozygites* and *Strongwellsea*.

Pseudocystidia are also named cystidia or paraphyses but they differ from basidiomycete cystidia and therefore the name pseudocystidia is preferred (Waterhouse, 1973). Similarly to sporophores, these structures break through the insect cuticle but pseudocystidia do not produce spores. They are considered to breach the insect cuticle and thus facilitate emergence of the sporophores (Brobyn & Wilding, 1977). Pseudocystidia occur only in *Erynia*, *Furia*, *Orthomyces*, *Pandora* and *Zoophthora* and occasionally in *Conidiobolus.* They are not used to distinguish genera.

Besides these structural features, characteristics of the parasitism or pathogenicity and life cycle of Entomophthorales have also been considered. This was for example the basis of the original distinction of the saprobically growing *Conidiobolus* species from the insect parasitic *Entomophthora*. The genus *Massospora* is characterized by the passive discharge of the primary spores that are released as the abdominal segments of the infected but still living cicada break off (White & Lloyd, 1983). *Strongwellsea* is well known for the hole that it forms on the ventral side of the abdomen of attacked diptera and through which spores are ejected while the insect is still flying (Strong *et al.*, 1960). Localized sporulation from the living host is also known in *Entomophthora thripidum* (Samson *et al.*, 1979) and *E. erupta* (Dunstan, 1924). Nevertheless, these features of the pathobiology and life cycle do not serve as taxon-defining characteristics.

Of all the morphological characters described, the structure of the sporophore, the mechanism of spore discharge and the morphological features of the primary spores seem to be accepted as taxonomic criteria on the generic level.

1.1.3 *QUANTITATIVE MORPHOLOGICAL CHARACTERISTICS ARE USED TO DISTINGUISH DIFFERENT SPECIES*

The taxonomic characters described so far are mostly being used to define the different genera of entomophthoralean fungi. Those characteristics that are not considered as valid on the generic level serve to separate groups within a specific genus. The features that can be meaningful at this level are formation and structure of rhizoids and pseudocystidia, morphology of primary spores, the types of secondary spores that are produced, resting spores and the distinction between parasitic and saprobic species (summarized in Ben-Ze'ev & Kenneth, 1982).

For the distinction to the species level quantitative morphological features, biochemical characteristics and the host-pathogen association are important (Remaudière *et al.*, 1976; Remaudière *et al.*, 1979; Ben-Ze'ev & Kenneth, 1982; Keller, 1993; Keller *et al.*, 1999):

- Precise shape and dimensions (length, width, ratio of both) of all types of spores.
- Structure and sizes of vegetative cells, sporophores, rhizoids or pseudocystidia.
- Qualitative and quantitative biochemical molecular characters (employed very rarely).
- Host range, characteristics of the host-pathogen interaction, behaviour of infected host and appearance of cadaver.

The characteristic features of the different genera belonging to the families Ancylistaceae, Entomophthoraceae and Neozygitaceae are summarized in Table 1. The classification is based on the taxonomy described by Humber (1989) that distinguishes the three genera *Erynia*, *Furia* and *Pandora*. The distinguishing structures for this separation are the rhizoids. *Tarichium* is only known from the resting spore stage and is therefore put in a provisional group and not defined with respect to the characteristic features used for the other groups (MacLeod & Müller-Kögler, 1970). The table was created by compilating informations mostly found in the following references: Humber, 1976; Keller, 1987; Humber, 1989; Keller, 1991; Balazy, 1993; Keller, 1997; Keller *et al.*, 1999.

Table 1: Summary of the characteristic features of entomophthoralean genera. NNPS = Number of nuclei in primary spores; SDM = Spore discharge mechanism; SP = Sporophore; SW = Spore wall (of primary spore); PS = primary spore. *: Humber (1989) describes the sporophores of *Erynia* as simple which is not in agreement with other authors (for example Keller, 1991; Balazy, 1993).

Family	Genera	SP	SW	NNPS		Spore morphology	SDM	Sec. Spore	Rhizoids	Pseudo-cystidia
Ancylistaceae	*Conidiobolus*	simple	unitunicate	plurinucleate	> 20	globose, ovoid, spherical	spore rounds off	as PS., microspores capillispores	present or absent	very rare
Neozygitaceae	*Neozygites*				4-8	globose, spherical, pyriform, ovoid	rounding off	as primary spores, capillispores	usually absent	absent
Entomophthoraceae	*Batkoa*				>2	globose, subglobose	rounding off	as primary spore	occasionally present	absent
	Entomophaga				>10	subglobose, pyriform	rounding off	as primary spore	absent	absent
	Entomophthora				≥2	bell shaped	SP explosion	as primary spore	present or absent	usually absent
	Eryniopsis				2-10	elongate, cylindrical, fusoid, pyriform	rounding off	as PS, capillispores on thick sporophore	present or absent	absent
	Massospora				2-8	long, elipsoid, ovoid, fusiform	passive	?	absent	?
	Erynia	*	bitunicate	uninucleate	1	elongate, fusoid, pyriform	rounding off	as primary spore, capillispores	present, thick no holdfast	
	Furia	branched			1	ovoid, cylindrical, fusoid	rounding off	as primary spore	not thicker than sporophore	
	Pandora				1	ovoid, cylindrical, fusoid	rounding off	as primary spore	2-3x thicker than sporophore prominent holdfast	
	Zoophthora				1	elongate, cylindrical, fusiform	rounding off	as primary spore, capillispores	rhizomorph	rare or absent
	Orthomyces				1	obovate	rounding off	as primary spore, capillispores	present or absent no holdfast	
	Strongwellsea				1	obovate, cone-shaped	rounding off	spherical, formed as primary spores	absent	
	Tarichium	The genus *Tarichium* is only known from resting spores.								

1.2 FROM OBLIGATE BIOTROPHIC PATHOGENS TO MYCO-INSECTICIDES? THE *IN VIVO* AND *IN VITRO* LIFE CYCLE OF ENTOMOPHTHORALES

The original incentive behind this project and behind research on Entomophthorales in general was the practical application of these fungi as biological pesticides. The initially defined goal of this work was to determine the potential of Entomophthorales for biocontrol and to evaluate the feasibility of an employment as biological insecticides. For these studies two entomophthoralean pathogens of thrips (Thysanoptera), *Neozygites parvispora* and *Entomophthora thripidum*, were investigated. These two species, like the majority of entomophthoralean fungi, are obligately biotrophic and very difficult to isolate, cultivate and manipulate under laboratory conditions. The distinct stages of the *in vivo* life cycle of many Entomophthorales are well described but it is difficult to control and induce different stages in the laboratory. Therefore, the reproduction of their life cycle under laboratory conditions is most challenging and crucial and provides the first hurdle for the utilization of Entomophthorales as mycoinsecticides.

1.2.1 THE IN VIVO *LIFE CYCLE OF ENTOMOPHTHORALES*

Most Entomophthorales are obligate biotrophic pathogens of insects or mites. The only substrate where these fungi usually grow is within the body of living insects (or mites). Initially, the pathogens colonize the hemocoel and later the fat body and central nervous system are invaded (Brobyn & Wilding, 1977). The gut, tracheae and cuticular structures are normally not attacked (Brobyn & Wilding, 1977). Entomophthoralean fungi multiply in the form of protoplasts, hyphal bodies or coenocytic mycelium (Fig. 1: ①). Growth as protoplasts is a peculiarity of Entomophthorales and has been found in a variety of species (Tyrell & MacLeod, 1972; Butt *et al.*, 1981; Nolan, 1985). It enables these fungi to evade the host's immune response (Beauvais *et al.*, 1989). Hyphal bodies, fragments of hyphae formed by budding or division, occur during vegetative growth of most insect pathogenic fungi (Prasertphon & Tanada, 1968). During invasion of the host Entomophthorales switch between segmented hyphal bodies or protoplasts and filamentous growth. The phase of vegetative growth usually ends when the host's body is packed with fungal cells. Only in the genus *Conidiobolus* toxins kill the host before extensive fungal development (Yendol *et al.*, 1968; Prasertphon & Tanada, 1969; Claydon, 1978; review in Roberts & Humber, 1981). In *Entomophaga maimaiga* and *E. aulicae* (= *E. egressa*) proteinaceous cell-lytic and toxic substances have been characterized (Dunphy

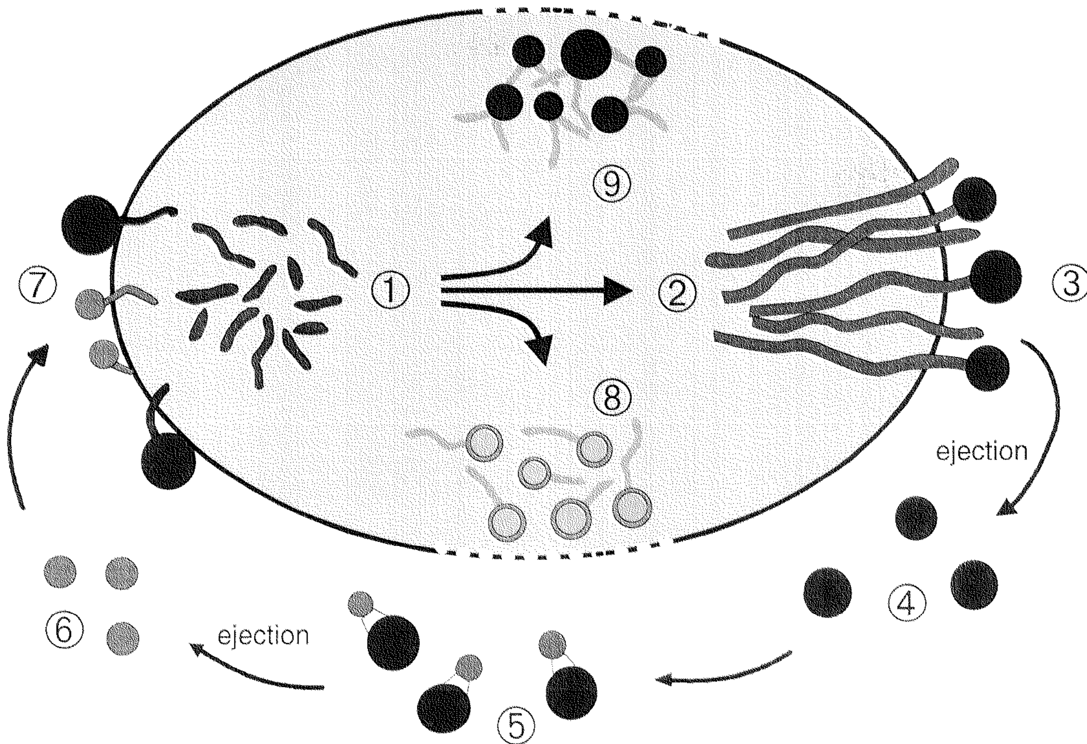


Fig. 1: Schematic life cycle of entomophthoralean fungi. Vegetative cells (①) grow and multiply only within the insect (turquois oval shape). Hyphae (②) break through the insect cuticle (③). The only structures occurring exclusively outside the insect are the primary (④) and secondary spores (⑤ and ⑥) that invade new hosts (⑦). Entomophthoralean fungi also form asexual (⑧) or sexual (⑨) resting stages.

& Nolan, 1982; Milne *et al.*, 1994). In general there seems to be an evolutionary trend towards slower and more localized invasion exemplified by the genera *Strongwellsea* or *Entomophthora*, as opposed to vigorous growth and fast killing due to the production of toxins (Humber, 1984).

The period of vegetative growth is followed by the switch to coordinated outward growth and breaking through the host cuticle (Fig. 1: ②). This differentiation leads to the formation of distinct structures like sporophores, pseudocystidia or rhizoids and usually occurs after the host has died. Once the entomophthoralean fungus has broken through the insect's cuticle spores are formed and actively ejected (Fig. 1: ③ and ④). The only exception herefore is the genus *Massospora*, where the active discharge mechanism was lost secondarily, but sporulation still occurs from living (and flying) cicada (Evans, 1989). Similarly, insects colonized by *Strongwellsea castrans* (Strong *et al.*, 1960), *Entomophthora thripidum* (Samson *et al.*, 1979) or *E. erupta* (Dunstan, 1924) are still alive when the fungus breaks through the cuticle and forms actively ejected primary spores. These primary spores (Fig. 1: ④) can form secondary or even higher order spores (Fig. 1: ⑤ and ⑥). If the infectious spore lands on a host insect it will germinate and penetrate the cuticle by mechanical force (Butt *et al.*, 1981) or by lysis of the insect's chitin shell with the help of

secreted proteases, lipases and chitinases (Fig. 1: ⑦) (Gabriel, 1968a, b; Latgé *et al.*, 1984; Samuels *et al.*, 1990; Urbanczyk *et al.*, 1992). Following penetration the fungus releases protoplasts or hyphal bodies and invades the insect body again, thus closing the vegetative cycle. Alternatively to this infectious cycle, entomophthoralean fungi can enter a dormant stage through the formation of azygo- or zygospores (Fig. 1: ⑧ and ⑨), that withstand periods during which the host is not available. These structures are thick walled and darkish colored and form either asexually (azygospores; Fig. 1: ⑧) or sexually (zygospores; Fig. 1: ⑨). Differentiation and induction of resting spores seems to be regulated by defined environmental stimuli (Brefeld, 1877; MacLeod *et al.*, 1973; Hajek & Shimazu, 1996; Hajek & Humber, 1997). The fungus remains in the dead insect, hidden in the soil or in vegetation, until the next host generation appears. After this quiescent period resting spores germinate and form infectious spores. In *Entomophthora muscae* resting spores germinated after partial degradation of their cell wall by chitinases produced by soil inhabiting bacteria (Schweizer, 1948).

1.2.2 IN VITRO *ISOLATION, CULTIVATION AND SPORULATION OF ENTOMOPHTHORALEAN PATHOGENS*

Under laboratory conditions the life cycle of Entomophthorales follows the same stages as *in vivo*. After isolation from a host the vegetative form of the fungus multiplies in artificial culture media. If suitable conditions and the right stimuli are provided the vegetatively growing cells will produce spores. These spores can be employed in a bioassay to infect the original host insect, which closes the vegetative life cycle of these pathogens. Therefore the ability to reproduce the life cycle under controlled conditions is a prerequisite for experimental studies as well as a practical application of these fungi. However, due to the obligate biotrophic habit of Entomophthorales the cultivation and induction of these different stages is exceptionally difficult.

1.2.2.1 Isolation

The isolation of an entomophthoralean pathogen from its insect host can be achieved by two different techniques: from freshly ejected primary spores that are collected in liquid or on solid medium (Sawyer, 1931; Schweizer, 1948) or by surface sterilization of infected host insects and the consecutive opening of these insects in artificial medium (MacLeod, 1956). The improvement especially of the latter method (better surface sterilization, isolation in small starting volumes and more complex media) made possible the successful isolation of several entomophthoralean pathogens (Leite *et al.*, 1996; Grundschober *et al.*,

1998; Freimoser *et al.*, 2000; Leite *et al.*, 2000). For highly specialized pathogens direct isolation of vegetatively growing cells is in general the method of choice because spores germinate very inefficiently in or on artificial media (Humber, 1994).

1.2.2.2 Cultivation

The first time that insect pathogenic Entomophthorales were cultivated was just 100 years ago when Brefeld managed to grow the vegetative state of *Entomophthora muscae* and soon afterwards Speare succeeded in reproducing the complete life cycle of an entomophthoralean pathogen under laboratory conditions (cited in Sawyer, 1929). In these early experiments such extraordinary substrates as swordfish, salmon or "meat water" and blood were employed (Sawyer, 1929; Schweizer, 1948). These rather special media were simplified by Müller-Kögler (1959) and the substrates that won recognition are egg-yolk and milk.

Nowadays Sabouraud-dextrose agar enriched with egg yolk or milk are the most frequently used culture media (Keller, 1987; Papierok & Hajek, 1997). On these substrates many Entomophthorales grow and members of the genus *Conidiobolus* are even less choosy and develop readily without the addition of egg yolk or milk (Keller, 1987). But on the other hand there are many species that require even richer media. Among this group are the genera *Entomophthora*, *Entomophaga* or *Neozygites.* Members of these genera are usually grown in complex liquid culture media that are either based on Grace's insect cell culture medium (Grace, 1962) or on the simpler GLEN medium (Beauvais & Latgé, 1988). Grace's insect cell culture medium is a chemically defined medium containing all common amino acids, several carbohydrates, salts and vitamins (see p. 68), while GLEN constitutes a complex mixture of lactalbumin hydrolysate, yeastolate, glucose and salt. These media are usually supplemented with FBS. Most difficult to please among all Entomophthorales that can be cultivated so far is *Neozygites parvispora*, which only grows in the presence of insect hemolymph and FBS (Grundschober *et al.*, 1998; see also chapter 4). The aphid pathogenic fungus *Neozygites fresenii* still can not be grown *in vitro* despite the numerous attempts and the rich media supplied.

Once the problem of the first isolation and the establishment of a continuously growing culture was overcome the optimal growth conditions of Entomophthorales were studied. The most thorough and detailed attempts to define the cultivation of Entomophthorales in artificial culture are again presented in the papers by Sawyer (1929) and Gustafsson (1965).

The following generalized statements can be made:

- Entomophthorales can not assimilate nitrate and many species require complex organic nitrogen sources.
- Entomophthorales do not produce invertase activity and can therefore not utilize sucrose as a carbon source.
- Most species grow best at temperatures between 18 and 24°C with the optimum around 20°C.
- Entomophthorales grow best at a slightly acidic pH between 6.5 and 7.

1.2.2.3 Sporulation

The distinction of entomophthoralean species that are easily cultivated from those with demanding requirements for *in vitro* cultivation is also expressed in the easiness to obtain spores *in vitro*. Generally Entomophthorales that grow easily sporulate spontaneously, while species that are difficult to grow require special conditions to induce sporulation.

Conidiobolus obscurus *forms resting spores in liquid media:--* Among all Entomophthorales the nutrient requirements of *Conidiobolus* seem to be met the easiest and *C. obscurus* was studied extensively as a potential mycoinsecticide. This species grows in complex liquid media as well as in defined mixtures containing glucose, amino acids and vitamins and readily forms resting spores in liquid culture (Latgé, 1975; Latgé & Remaudière, 1975; Latgé, 1977; Latgé *et al.*, 1978; Perry & Latgé, 1980; Latgé & Sanglier, 1985). These durable spores were envisioned as the form most suitable for biocontrol, and methods for large-scale production of resting spores have been developed (Remaudière, 1971; Latgé, 1977). This is in contrast to other "easily growing" Entomophthorales where the production and formulation of spores is much more difficult. In such cases, like *Pandora neoaphidis* or *Zoophthora radicans*, mycelium is applied directly and infectious spores should be formed and ejected in the field (Li *et al.*, 1993; Shah *et al.*, 1999).

"Difficult" Entomophthorales have to switch to hyphal growth prior to sporulation:-- More difficult to "tame" are the species belonging to the genera *Entomophaga*, *Entomophthora* or *Neozygites* (Latgé, 1981). One of the best studied species in this category of "difficult" fungi is *Entomophaga aulicae* which develops through different protoplast stages prior to the formation of walled structures (Nolan, 1985; Nolan, 1987). In *E. aulicae* the differentiation process from protoplasts to cells surrounded by a wall has been studied in various ways by changing environmental or cultivation conditions, the composition of the

medium or by including charged surfaces in the growth flask (Nolan, 1985; Beauvais & Latgé, 1988; Nolan, 1991). A similar differentiation from protoplasts to walled hyphae occurs in *Entomophthora thripidum* (Freimoser *et al.*, 2000). For *E. thripidum* as well as for *N. parvispora* hyphal growth is a prerequisite for sporulation (Freimoser *et al.*, 2000; Grundschober, 2000; see also chapter 6). After cell wall formation and the induction of hyphal growth these species will sporulate spontaneously if brought onto a solid surface; just as the "easier growing" species do sporulate spontaneously.

With the formation of spores the life cycle of the fungus is almost complete. It has been shown repeatedly that artificially produced spores are infectious to the original host insects (Sawyer, 1929; Eilenberg *et al.*, 1990; Grundschober, 2000; see also chapter 6). With the consecutive reisolation of the fungus the life cycle is completed and the pathogenic nature of the fungus in question is proven as Bassi had already achieved for *Beauveria bassiana* more than 100 years ago.

1.2.2.4 Field experiences with entomophthoralean pathogens

All this work investigating the isolation, cultivation and control of the life cycle of Entomophthorales *in vitro* was mainly motivated by the prospect of an application as mycoinsecticides. Unfortunately, field trials and attempts for a commercial utilization have not been successful so far. However, the entomophthoralean pathogen *Zoophthora radicans* was established and successfully controlled aphids in a classical biocontrol program in Australia (Milner *et al.*, 1982). *Zoophthora radicans* was released in southern Australia and caused high mortality within short time despite the lack of rainfall and the dry climate (Milner *et al.*, 1982). Another well-documented example for the effectiveness of entomophthoralean fungi is the case of *Entomophaga maimaiga*. This pathogen attacks *Lymantria dispar* (gypsy moth) that was accidentally introduced in the USA about 130 years ago (Hajek, 1999). Much later, in 1989, *E. maimaiga* was first found to cause high mortality in *L. dispar* and the fungus then rapidly spread and followed its host (reviewed in Hajek, 1999).

These encouraging examples as well as the plentiful reports of natural epizootics caused by Entomophthorales clearly show the effectiveness and potential of these fungi. But it is also apparent that it is not really known why, how and when they "work". Therefore a more detailed knowledge of the life cycle of Entomophthorales envisioned as putative micoinsecticides is required.

REFERENCES

Ainsworth, G. C. (1956). Agostino Bassi, 1773-1856. *Nature* **177**, 255-257.

Balazy, S. (1993). *Flora of Poland, Fungi (Mycota) no. 24: Entomophthorales.*

Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Ann Rev Microbiol* **22**, 87-108.

Beauvais, A. & Latgé, J. P. (1988). A simple medium for growing entomophthoralean protoplasts. *J Invertebr Pathol* **51**, 175-178.

Beauvais, A., Latgé, J. P., Vey, A. & Prevost, M. C. (1989). The role of surface components of the entomopathogenic fungus *Entomophaga aulicae* in the cellular immune response of *Galleria mellonella* Lepidoptera. *J Gen Microbiol* **135**, 489-498.

Ben-Ze'ev, I. & Kenneth, R. G. (1982). Features-criteria of taxonomic value in the Entomophthorales: I. A revision of the Batkoan classification. *Mycotaxon* **14**, 393-455.

Ben-Ze'ev, I., Kenneth, R. G. & Uziel, A. (1987). A reclassification of *Entomophthora turbinata* in *Thaxterosporium* gen. nov., Neozygitaceae fam. nov. (Zygomycetes: Entomophthorales). *Mycotaxon* **28**, 313-326.

Brefeld, O. (1877). Ueber die Entomophtoreen und ihre Verwandten. *Bot Zeitung* **22**, 345-355.

Brobyn, P. J. & Wilding, N. (1977). Invasive and developmental processes of *Entomophthora* species infecting aphids. *Trans Brit Mycol Soc* **69**, 349-366.

Butt, T. M., Beckett, A. & Wilding, N. (1981). Protoplasts in the *in vivo* life cycle of *Erynia neoaphidis*. *J Gen Microbiol* **127**, 417-421.

Cavalier-Smith, T. (1987). The origin of fungi and pseudofungi. In *Evolutionary Biology of the Fungi*, pp. 339-353. Edited by Rayner, A. D. M., Brasier, C. M., & Moore, D. M. Cambridge: Cambridge University Press.

Cavalier-Smith, T. (1998). A revised six-kingdom system of life. *Biol Rev* **73**, 203-266.

Claydon, N. (1978). Insecticidal secondary metabolites from entomogenous fungi: *Entomophthora virulenta*. *J Invertebr Pathol* **32**, 319-324.

Cohn, F. (1855a). *Empusa muscae* und die Krankheit der Stubenfliegen. *Verhandl Leop-Carol Acad Naturforsch* **25**, 299-360.

Cohn, F. (1855b). *Empusa muscae* und die Krankheit der Stubenfliegen. *Hedwigia* **10**, 57-61.

Coulson, J. R., Klaasen, W., Cook, R. J., King, E. G., Chiang, H. C., Hagen, K. S. & Yendol, W. G. (1982). Notes on biological control of pests in China, 1979. In *Biological Control of Pests in China*, pp. 1-192. Washington, DC: US Department of Agriculture.

Crosby, A. W. (1996). *Ecological imperialism: The biological expansion of Europe 900-1900*, (first Edition 1986) edn. Cambridge: Cambridge Univeristy Press.

Debach, P. & Rosen, D. (1991). *Biological control by natural enemies*, 2nd edn. Cambridge: Cambridge University Press.

Dunphy, G. B. & Nolan, R. A. (1982). Mycotoxin production by the protoplast stage of *Entomophthora egressa*. *J Invertebr Pathol* **39**, 261-263.

Dunstan, A. G. (1924). Studies on a new species of *Empusa* parasitic on the green apple bug (*Lygus communis* var. *novascotiensis* Knight) in the Annapolis Valley. *Proc Acadian Entomol Soc for 1923* **9**, 14-36.

Eilenberg, J., Bresciani, J. & Latgé, J. P. (1986). Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora*. *J Invertebr Pathol* **48**, 318-324.

Eilenberg, J., Bresciani, J. & Latgé, J. P. (1990). Primary spore and resting spore formation *in vitro* of *Entomophthora schizophorae* and *Entomophthora muscae*, both members of the *Entomophthora muscae* complex (Zygomycetes). *Cryptogamic Bot* **1**, 365-371.

Eilenberg, J., Bresciani, J., Olesen, U. & Olson, L. (1995). Ultrastructural studies of secondary spore formation and discharge in the genus *Entomophthora*. *J Invertebr Pathol* **65**, 179-185.

Evans, H. C. (1989). Mycophathogens of insects of epigeal and aerial habitats. In *Insect-Fungus Interactions*, pp. 205-238. Edited by Wilding, N., Collins, N. M., Hammond, P. M., & Webber, J. F. London: Academic Press.

Freimoser, F. M., Grundschober, A., Aebi, M. & Tuor, U. (2000). *In vitro* cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements, and sporulation. *Mycologia* **92**, 208-215.

Fresenius, G. (1856). Notiz, Insekten-Pilze betreffend. *Bot Zeitung* **14**, 882-883.

Gabriel, B. P. (1968a). Enzymatic activity of some entomophthorous fungi. *J Invertebr Pathol* **11**, 70-81.

Gabriel, B. P. (1968b). Histochemical study of the insect cuticle infected by the fungus *Entomophthora coronata*. *J Invertebr Pathol* **11**, 82-89.

Goethe, J. W. v. (1820). Verstäubung, Verdunstung, Vertropfung. In *Hefte zur Morphology*.

Grace, T. D. C. (1962). Establishment of four strains of cells from insect tissues grown *in vitro*. *Nature* **195**, 788-789.

Grundschober, A., Tuor, U. & Aebi, M. (1998). *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *Syst Appl Microbiol* **21**, 461-469.

Gugnani, H. C. (1999). A review of zygomycosis due to *Basidiobolus ranarum*. *Eur J Epidemiol* **15**, 923-929.

Grundschober, A. (2000). *In vitro* life cycle of *Neozygites parvispora* and *Entomophthora thripidum*, two fungal pathogens of *Thrips tabaci*. In *Institute of Microbiology*, pp. 127. Zurich: ETH Zurich.

Gustafsson, M. (1965). On species of the genus *Entomophthora* Fres. in Sweden. I. Classification and Distribution. *Lantbrukshögskolans Annaler* **31**, 103-212.

Hajek, A. E. (1999). Pathology and epizootiology of *Entomophaga maimaiga* infections in forest lepidoptera. *Microbiol Mol Biol Rev* **63**, 814-835.

Hajek, A. E. & Humber, R. A. (1997). Formation and germination of *Entomophaga maimaiga* azygospores. *Can J Bot* **75**, 1739-1747.

Hajek, A. E. & Shimazu, M. (1996). Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth *Lymantria dispar*. *Can J Bot* **74**, 708-715.

Hall, I. M. & Bell, J. V. (1962). Nomenclature of *Empusa* Cohn 1855 vs. *Entomophthora* Fresenius 1856. *J Insect Pathol* **4**, 224-228.

Hesseltine, C. W. & Ellis, J. J. (1973). Mucorales. In *The Fungi, an advanced treatise*, pp. 187-217. Edited by Ainsworth, G. C., Sparrow, F. K., & Sussman, A. S. NY: Academic Press.

Hoddinott, J. & Olsen, O. A. (1972). A study of the carbohydrates in the cell walls of some species of the Entomophthorales. *Can J Bot* **50**, 1675-1679.

Hoffmann, W. E. (1947). Insects as human food. *Proc Entomol Soc Washington* **49**, 233-237.

Humber, R. A. (1976). The systematics of the genus *Strongwellsea* (Zygomycetes: Entomophthorales). *Mycologia* **68**, 1042-1060.

Humber, R. A. (1981). An alternative view of certain taxonomic criteria used in Entomophthorales (Zygomycetes). *Mycotaxon* **13**, 190-240.

Humber, R. A. (1984). Foundations for an evolutionary classification of the Entomophthorales (Zygomycetes). In *Fungus-Insect Relationships: Perspectives In Ecology and Evolution*, pp. 166-183. Edited by Wheeler, Q. & Blackwell, M. New York, N.Y., USA: Columbia University Press.

Humber, R. A. (1989). Synopsis of a revised classification for the Entomophthorales (Zygomycotina). *Mycotaxon* **34**, 441-460.

Humber, R. A. (1994). Special considerations for operating a culture collection of fastidious fungal pathogens. *J Indust Microbiol* **13**, 195-196.

Humber, R. A. & Feng, M. G. (1991). *Entomophthora chromaphidis* (Entomophthorales): The correct identification of an aphid pathogen in the Pacific Northwest and elsewhere. *Mycotaxon* **41**, 497-504.

Hutchison, J. A. (1963). The genus *Entomophthora* in the western hemisphere. *Trans Kansas Acad Sci* **66**, 237-254.

Ingold, C. T. (1934). The spore discharge mechanism in *Basidobolus ranarum*. *New Phytologist* **33**, 274-277.

Ingold, C. T. (1953). *Dispersal in Fungi*. Oxford: Clarendon Press.

Ingold, C. T. (1966). Spore release. In *The Fungi, an advanced treatise*, pp. 679-707. Edited by Ainsworth, G. C. & Sussman, A. S. NY: Academic Press.

Jensen, A. B., Gargas, A., Eilenberg, J. & Rosendahl, S. (1998). Relationships of the insect-pathogenic order Entomophthorales (Zygomycota, Fungi) based on the phylogenetic analysis of small ribosomal sequences (SSU rDNA). *Fungal Genet Biol* **24**, 325-334.

Keller, S. (1987). Arthropod-pathogenic Entomophthorales of Switzerland. I *Conidiobolus*, *Entomophaga* and *Entomophthora*. *Sydowia* **40**, 122-167.

Keller, S. (1991). Arthropod-pathogenic Entomophthorales of Switzerland: II. *Erynia*, *Eryniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*. *Sydowia* **43**, 39-122.

Keller, S. (1993). Taxonomic considerations on some species of *Erynia* (Zygomycetes, Entomophthorales) attacking flies (Diptera). *Sydowia* **45**, 252-263.

Keller, S. (1997). The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. *Sydowia* **49**, 118-146.

Keller, S. & Eilenberg, J. (1993). Two new species of Entomophthoraceae (Zygomycetes, Entomophthorales) linking the genera *Entomophaga* and *Eryniopsis*. *Sydowia* **45**, 264-274.

Keller, S., Kalsbeek, V. & Eilenberg, J. (1999). Redescription of *Entomophthora muscae* (Cohn) Fresenius. *Sydowia* **51**, 197-209.

Kobayasi, Y. (1977). Miscellaneous notes on the genus *Cordyceps* and its allies. *J Jap Bot* **52**, 269-272.

Latgé, J. P. (1975). Croissance et sporulation de 6 espèces d'Entomophthorales. 1. Influence de la nutrition carbonée. *Entomophaga* **20**, 201-207.

Latgé, J. P. (1977). Croissance et sporulation d'*Entomophthora virulenta* Hall et Dunn en culture liquide discontinue. *C R Acad Sc, Paris t 284 Série D* , 1879-1882.

Latgé, J. P. (1981). Nutritional requirements of Entomophthorales. *Ann Microbiol (Inst Pasteur)* **132**, 299-306.

Latgé, J. P. & Remaudière, G. (1975). Growth and sporulation of six species of Entomophthorales. III. Influence of concentrations of carbon and nitrogen and the carbon/nitrogen ratio. *Revue de Mycologie* **39**, 239-250.

Latgé, J. P., Remaudière, G., Soper, R. S., Madore, C. D. & Diaquin, M. (1978). Growth and sporulation of *Entomophthora virulenta* on semidefined media in liquid culture. *J Invertebr Pathol* **31**, 225-233.

Latgé, J. P., Sampedro, L. & Hall, R. (1984). Aggressiveness of *Conidiobolus obscurus* towards the pea aphid. III. Extracellular enzymatic activities. *Entomophaga* **29**, 185-201.

Latgé, J. P. & Sanglier, J. J. (1985). Optimisation de la croissance et de la sporulation de *Conidiobolus obscurus* en milieu défini. *Can J Bot* **63**, 68-85.

Latgé, J. P., Soper, R. S., Madore, C. D. (1977). Media suitable for industrial production of *Entomophthora virulenta* zygospores. *Biotech Bioeng* **19**, 1269-1284.

Leite, L. G., Smith, L., Moraes, G. J. & Roberts, D. W. (1996). Production of hyphal bodies of the fungus *Neozygites* nr. *floridana "in vitro"*. In *V Siconbiol. Simposio de controle biologico*, pp. 448. Edited by EMBRAPA. Parana Brasil.

Leite, L. G., Smith, L., Moraes, G. J. & Roberts, D. W. (2000). *In vitro* production of hyphal bodies of the mite pathogenic fungus *Neozygites floridana*. *Mycologia* **92**, 201-207.

Li, Z., Butt, T. M., Beckett, A. & Wilding, N. (1993). The structure of dry mycelia of the entomophthoralean fungi *Zoophthora radicans* and *Erynia neoaphidis* following different preparatory treatments. *Mycol Res* **97**, 1315-1323.

MacLeod, D. M. (1956). Notes on the genus *Empusa* Cohn. *Can J Bot* **34**, 16-26.

MacLeod, D. M. & Müller-Kögler, E. (1970). Insect pathogens: species originally described from their resting spores mostly as *Tarichium* species (Entomophthorales: Entomophthoraceae). *Mycologia* **62**, 33-66.

MacLeod, D. M., Müller-Kögler, E. & Wilding, N. (1976). *Entomophthora* species with *E. muscae*-like conidia. *Mycologia* **68**, 1-29.

MacLeod, D. M., Tyrrell, D., Soper, R. S. & De Lyzer, A. J. (1973). *Entomophthora bullata* as a pathogen of *Sarcophaga aldrichi*. *J Invertebr Pathol* **22**, 75-79.

Martin, G. W. (1925). Morphology of *Conidiobolus villosus*. *Bot Gazette* **80**, 311-318.

Milne, R., Wright, T., Welton, M., Budau, C., Gringorten, L. & Tyrrell, D. (1994). Identification and partial purification of a cell-lytic factor from *Entomophaga aulicae*. *J Invertebr Pathol* **64**, 253-259.

Milner, R. J., Soper, R. S. & Lutton, G. G. (1982). Field release of an Israeli strain of the fungus *Zoophthora radicans* (Brefeld) Batko for the biological control of *Therioaphis trifolii* (Monell) f. *maculata*. *J Aust Entomol Soc* **21**, 113-118.

Müller-Kögler, E. (1959). Zur Isolierung und Kultur insektenpathogener Entomophthoraceen. *Entomophaga* **4**, 261-274.

Nagahama, T., Sato, H., Shimazu, M. & Sugiyama, J. (1995). Phylogenetic divergence of the entomophthoralean fungi: evidence from nuclear 18S ribosomal RNA gene sequences. *Mycologia* **87**, 203-209.

Nolan, R. A. (1985). Protoplasts from Entomophthorales. In *Fungal Protoplasts*, pp. 87-112. Edited by Peberdy, J. F. & Ferenczy, L. New York: Marcel Dekker Inc.

Nolan, R. A. (1987). Microstage development for protoplasts of the fungus *Entomophaga aulicae*. *Can J Microbiol* **33**, 808-811.

Nolan, R. A. (1991). Influence of a negatively charged surface (Teflon disk) on *Entomophaga aulicae* protoplast morphogenesis under mass fermentation conditions. *Can J Bot* **69**, 2578-2581.

Papierok, B. & Hajek, A. E. (1997). Fungi: Entomophthorales. In *Manual of Techniques in Insect Pathology*, pp. 187-212. Edited by Lacey, L. San Diego: Academic Press.

Perry, D. F. & Latgé, J. P. (1980). Chemically defined media for growth and sporulation of *Entomophthora virulenta*. *J Invertebr Pathol* **35**, 43-48.

Prasertphon, S. & Tanada, Y. (1968). The formation and circulation, in *Galleria*, of hyphal bodies of entomophthoraceous fungi. *J Invertebr Pathol* **11**, 260-280.

Prasertphon, S. & Tanada, Y. (1969). Mycotoxins of Entomophthoraceous fungi. *Hilgardia* **39**, 581-600.

Remaudière, G. (1971). Vers l'utilisation pratique des Entomophthorales parasites de pucerons. *Parasitica* **27**, 115-126.

Remaudière, G. & Hennebert, G. L. (1980). Systematic revision of *Entomophthora aphidis* Hoffm. in Fres. Description of two new aphid pathogens. *Mycotaxon* **11**, 269-321.

Remaudière, G. & Keller, S. (1980). Révision systématique des genres d'Entomophthoraceae à potentialité entomopathogène. *Mycotaxon* **11**, 323-338.

Remaudière, G., Keller, S., Papierok, B. & Latgé, J. P. (1976). Considérations systématiques et biologiques sur quelques espèces d'*Entomophthora* du groupe *sphaerosperma* pathogènes d'insectes (Phycomycetes: Entomophthoraceae). *Entomophaga* **21**, 163-177.

Remaudière, G., Latgé, J. P. & Papierok, B. (1979). Reconsidération taxonomique de *Entomophthora obscura* Hall et Dunn. *Ann Microbiol (Inst Pasteur)* **130A**, 151-162.

Roberts, D. W. & Humber, R. A. (1981). Entomogenous fungi. In *Biology of conidial fungi*, pp. 201-236: Academic Press.

Sailer, R. I. (1983). History of insect introductions. In *Exotic plant pests and north american agriculture*, pp. 15-38. Edited by Wilson, C. L. & Gaham, C. L. London: Academic Press.

Samson, R. A., Ramakers, P. M. J. & Oswald, T. (1979). *Entomophthora thripidum*, a new fungal pathogen of *Thrips tabaci*. *Can J Bot* **57**, 1317-1323.

Samuels, R. I., Charnley, A. K. & St. Leger, R. J. (1990). The partial characterization of endoproteases and exoproteases from three species of entomopathogenic Entomophthorales and two species of Deuteromycetes. *Mycopathologia* **110**, 145-152.

Sawyer, W. H. j. (1929). Observations on some entomogenous members of the Entomophthoraceae in artificial cultures. *Am J Botany* **16**, 87-121.

Sawyer, W. H. j. (1931). Studies on the morphology and development of an insect-destroying fungus, *Entomophthora sphaerosperma*. *Mycologia* **23**, 411-432.

Schweizer, G. G. (1948). Ueber die Kultur von *Empusa muscae* Cohn und anderen Entomophthoraceen auf kalt sterilisierten Nährböden. *Planta* **35**, 132-176.

Shah, P. A., Aebi, M. & Tuor, U. (1999). Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. *Biocontrol Sci Tech* **9**, 19-28.

Steinhaus, E. A. (1956). Microbial control-the emergence of an idea. *Hilgardia* **26**, 107-160.

Steinkraus, D. C., Oliver, J. B., Humber, R. A. & Gaylor, M. J. (1998). Mycosis of bandedwinged whitefly (*Trialeurodes abutilonea*) (Homoptera: Aleyrodidae) caused by *Orthomyces aleyrodis* gen. and sp. nov. (Entomophthorales: Entomophthoraceae). *J Invertebr Pathol* **72**, 1-8.

Strong, F. E., Wells, K. & Apple, J. W. (1960). An unidentified fungus parasitic on the seed-corn maggot. *J Econ Entomol* **53**, 478-479.

Thaxter, R. (1888). The Entomophthorae of the United States. *Mem Boston Soc Nat Hist* **4**, 133-201.

Tyrell, D. & MacLeod, D. M. (1972). Spontaneous formation of protoplasts by a species of *Entomophthora*. *J Invertebr Pathol* **19**, 354-360.

Urbanczyk, M. J., Zabza, A., Balazy, S. & Peczynskia-Czoch, W. (1992). Laboratory culture media and enzyme activity of some entomopathogenic fungi of *Zoophthora* (Zygomycetes Entomophthoraceae). *J Invertebr Pathol* **59**, 250-257.

Waterhouse, G. M. (1973). Entomophthorales. In *The Fungi, an advanced treatise*, pp. 219-229. Edited by Ainsworth, G. C., Sparrow, F. K., & Sussman, A. S. NY: Academic Press.

White, J. & Lloyd, M. (1983). A pathogenic fungus, *Massospora cicadina* Peck (Entomophthorales), in emerging nymphs of periodical cicadas (Homoptera: Cicadidae). *Environ Entomol* **12**, 1245-1252.

Wilding, N. & Brady, B. L. (1984a). *Conidiobolus obscurus.* In *Commonwealth Mycological Institute, Descriptions of pathogenic fungi and bacteria no. 812.* Aberystwyth, UK: The Cambrian News.

Wilding, N. & Brady, B. L. (1984b). *Entomophthora planchoniana.* In *Commonwealth Mycological Institute, Descriptions of pathogenic fungi and bacteria no. 814.* Aberystwyth, UK: The Cambrian News.

Wilding, N. & Brady, B. L. (1984c). *Erynia neoaphidis.* In *Commonwealth Mycological Institute, Descriptions of pathogenic fungi and bacteria no. 815.* Aberystwyth, UK: The Cambrian News.

Wilding, N. & Brady, B. L. (1984d). *Neozygites fresenii.* In *Commonwealth Mycological Institute, Descriptions of pathogenic fungi and bacteria no. 817.* Aberystwyth, UK: The Cambrian News.

Yendol, W. G., Miller, E. M. & Behnke, C. N. (1968). Toxic substances from entomophthoraceous fungi. *J Invertebr Pathol* **10**, 313-319.

CHAPTER 2

Phylogenetic analysis of SSU rDNA sequences suggests a common origin of Neozygitaceae (Zygomycota; Entomophthorales), fungal pathogens of arthropods, and of microsporidia, obligate intracellular parasites

Florian M. Freimoser
Urs Tuor
Markus Aebi[1]

Institute of Microbiology
ETH Zürich
Schmelzbergstr. 7
CH-8092 Zürich, Switzerland

Submitted for publication

[1]Corresponding author: Markus Aebi, Institute of Microbiology, ETH Zürich, Schmelzbergstr. 7, CH-8092 Zürich. Tel. ++41/+1/632'64'13. Fax: ++41/+1/632'11'48. e-mail: aebi@micro.biol.ethz.ch

Seite Leer /
Blank leaf

Phylogenetic analysis of SSU rDNA sequences suggests a common origin of Neozygitaceae (Zygomycota; Entomophthorales), fungal pathogens of arthropods, and of microsporidia, obligate intracellular parasites

Microsporidia are amitochondrial obligate intracellular parasites of invertebrates and vertebrates. They can occur as opportunistic pathogens in immunocompromised humans (Mathis, 2000). The phylogenetic ancestry of microsporidia is under debate: they were considered as ancient eukaryotes (Vossbrinck & Woese, 1986; Vossbrinck *et al.*, 1987; Kamaishi *et al.*, 1996) and classified in the archezoan subkingdom (Cavalier-Smith, 1983), but molecular data also suggest an evolutionary origin within the fungal kingdom (Cavalier-Smith, 1998; Keeling, 2000). The family of the Neozygitaceae belongs to the fungal order of the Entomophthorales (Zygomycota), which is comprised of many obligate biotrophic pathogens of arthropods. Neozygitaceae are known for their demanding requirements for continuous cultivation *in vitro* (Grundschober *et al.*, 1998; Leite *et al.*, 2000). Characteristically they form highly specialised spores (capilliconidia) that serve as infectious units to colonise the hemocoel of a new host (Keller, 1997). A phylogenetic analysis of this family at the molecular level based on SSU rDNA (small subunit ribosomal DNA) sequences revealed that Neozygitaceae and microsporidia formed sister groups within the Entomophthorales and localised for the first time the microsporidian origin within the fungal kingdom. This systematic assignment was supported further by striking similarities in the infectious structures of microsporidia and Neozygitaceae. Our analysis suggested that microsporidia represent the most highly evolved clade within the Entomophthorales and not ancient eukaryotes.

INTRODUCTION

The Entomophthorales represent an order within the fungal phylum of the Zygomycota and are comprised of many obligate biotrophic pathogens of insects and mites. In contrast to other Zygomycota, these fungi are

characterised by the presence of chitin and glucans in their cell walls (Hoddinott & Olsen, 1972). Nonmotile, actively ejected spores that represent single spored sporangia are used to infect new hosts (Thaxter, 1888; Hesseltine & Ellis, 1973; Cavalier-Smith, 1987). The two families Ancylistaceae and Entomophthoraceae contain the majority of the insect pathogenic species and form a monophyletic group (Nagahama *et al.*, 1995; Jensen *et al.*, 1998). The family of the Neozygitaceae also belongs to the Entomophthorales, but the phylogenetic relationship is not known. This family comprises important pathogens of mites, thrips or aphids (Carl, 1975; Steinkraus *et al.*, 1991; Delalibera *et al.*, 1992). Nuclear morphology and nuclear behaviour during mitosis are characteristics that are used to distinguish this family from other Entomophthorales (Ben-Ze'ev, 1987; Butt & Humber, 1989). In contrast to other Entomophthorales, species of the genus *Neozygites* are widely distributed in tropical regions and are also exceptional for the extremely demanding requirements for growth *in vitro* (Grundschober *et al.*, 1998; Leite *et al.*, 2000). The possibility to obtain pure *in vitro* cultures of Neozygitaceae enabled us to perform a phylogenetic analysis of this family at the molecular level based on SSU rDNA sequences.

For this analysis we included sequences of microsporidia because a weakly supported relationship of microsporidia with *Entomophaga aulicae* (Van de Peer *et al.*, 2000) and *Conidiobolus coronatus* (Entomophthorales) or Ascomycetes was recently reported (Keeling, 2000).

Microsporidia are obligate intracellular parasites of vertebrates and invertebrates (Mathis, 2000) and occur as opportunistic pathogens in immunocompromised humans. Furthermore, their potential use as biocontrol agents caused interest in their biological nature (Solter & Maddox, 1998). However, the taxonomic belonging of microsporidia is still a matter of debate. Originally, microsporidia were classified as protists based on molecular (Vossbrinck & Woese, 1986; Vossbrinck *et al.*, 1987; Kamaishi *et al.*, 1996) and structural characteristics such as the lack of mitochondria, peroxisomes, Golgi stacks, microbodies and flagella (Müller, 1997; Keeling, 1998). However, the discovery of a mitochondrian heat shock protein encoded in a microsporidian genome (Germot *et al.*, 1997; Hirt *et al.*, 1997; Peyretaillade *et al.*, 1998) as well as phylogenetic analysis based on the amino acid sequences of α- and β-tubulin (Edlind *et al.*, 1996; Keeling & Doolittle, 1996; Keeling, 2000), the TATA box binding protein (Fast *et al.*, 1999), the largest subunit of RNA polymerase II (Hirt *et al.*, 1999) and the sequence of the large subunit ribosomal DNA (Van de Peer *et al.*, 2000) disprove an ancient origin and suggest a fungal ancestry of microsporidia instead.

In our analysis of the SSU rDNA of Neozygitaceae, other Entomophthorales and microsporidia a close relationship of the Neozygitaceae- and microsporidia-sequences was discovered. In addition, the life cycle of both Neozygitaceae and microsporidia present striking similarities. It was therefore concluded that microsporidia have their origin within the phylum of the Zygomycota and belong to the Entomophthorales.

MATERIAL AND METHODS

The SSU rDNA of the following entomophthoralean species was partially sequenced: *Conidiobolus coronatus* (kindly provided by A. A. Callaghan, Staffordshire Univ., UK), 2 isolates of *C. obscurus* (ARSEF 133 and isolate 560 of our collection, kindly provided by S. Keller, FAL Reckenholz, CH), *C. lamprauges* (ARSEF 2338), *Neozygites parvispora* (ARSEF 6276), *N. floridana* (isolate 315 of our collection), *Neozygites sp.* (ARSEF 662) and *Entomophthora thripidum* (ARSEF 6518). DNA was extracted from freeze-dried mycelium or cells according to Zolan and Pukkila (1986). SSU rDNA was amplified by PCR using the primers nu-SSU-0021-5' (CTGGTTGATTCT-GCCAGT) or nu-SSU-0402-5' (CCGGAGAGGGAGCCTGAGAAAC) (for *N. parvispora*) and nu-SSU-1780-3' (AATGATCCTTCCGCAGGT) as described elsewhere (Jensen *et al.*, 1998). PCR products were sequenced on both strands using specific primers, ABI chemicals and the ABI PRISM 310 Genetic Analyzer.
All sequences determined for this study and previously published SSU rDNA sequences from other organisms (Tab. 1) were aligned using Clustal X (Thompson *et al.*, 1997). The alignment was manually corrected. All gaps and ambiguously aligned positions were excluded. As an outgroup *Acanthocoepis unguiculata* and *Diaphanoeca grandis* were used because they had proven suitable for this purpose (Jensen *et al.*, 1998).

Phylogenetic analysis was performed with PAUP version 4.0b4a (Swofford, 1999). Skewness of the tree-length distribution of 100'000 randomly generated trees was used to assess the strength of phylogenetic signal (Hillis & Huelsenbeck, 1992). Most parsimonious trees were determined by heuristic search with 500 replicates of random sequence additions and TBR branch swapping. 1000 bootstrap searches were performed with 10 random sequence additions each. Decay analysis was performed with unresolved monophyly constraint trees (heuristic search, random sequence addition, 500 replicates) (Bruns *et al.*, 1992; Groth & Barrowclough, 1999). Likelihood values were determined for all unconstrained and constrained most parsimonious trees. The

Table 1. Names and accession numbers of SSU rDNA sequences of species used in this study.

		SPECIES	ACCESSION NUMBER
Zygomycota			
	Entomophthorales	*Conidiobolus thromboides*	AF 052401
		Conidiobolus coronatus 1	AF 113417
		Conidiobolus coronatus 2	AF 296753
		Conidiobolus incongruus	AF 113419
		Conidiobolus lamprauges 1	AF 113420
		Conidiobolus lamprauges 2	AF 296754
		Conidiobolus obscurus 1	AF 296756
		Conidiobolus obscurus 2	AF 296757
		Entomophaga aulicae	U 35394
		Entomophthora schizophora	AF 052402
		Entomophthora thripidum	AF 296755
		Eryniopsis ptycoptorae	AF 052403
		Neozygites floridana	AF 296758
		Neozygites parvispora	AF 296760
		Neozygites sp.	AF 296759
		Pandora neoaphidis	AF 052405
		Strongwellsea castrans	AF 052406
		Zoophthora radicans	D 61381
Mucorales		*Mucor racemosus*	AF 113430
		Syncephalastrum racemosum	X 89437
	Glomales	*Glomus sinosum*	AJ 133706
	Harpellales	*Smittium culisetae*	AF 007540
Chytridiomycota			
		Basidiobolus ranarum	AF 113414
		Basidiobolus haptosporus	AF 113413
	Chytridiales	*Chytridium confervae*	M 59758
	Neocallimastigales	*Neocallimastix frontalis*	X 80341
	Spizellomycetales	*Spizellomyces acuminatus*	M 59759
Microsporidia			
		Encephalitozoon hellem	AF177920
		Glugea americanus	AF056014
		Vittaforma corneae	L39112
		Thelohania solenopsae	AF031538
		Amblyospora stimuli	AF027685
Choanoflagellida			
(outgroup)		*Acanthocoepsis unguiculata*	L 10823
		Diaphanoeca grandis	L 10824

most likely trees from parsimony analysis with and without constraints were compared using the Templeton (Wilcoxon signed rank) test. Maximum likelihood trees were calculated by heuristic search of 100 random sequence additions. Phylogenetic trees based on DNA distances were calculated by employing different substitution models using default settings and optionally with 100 bootstrap resamplings.

RESULTS

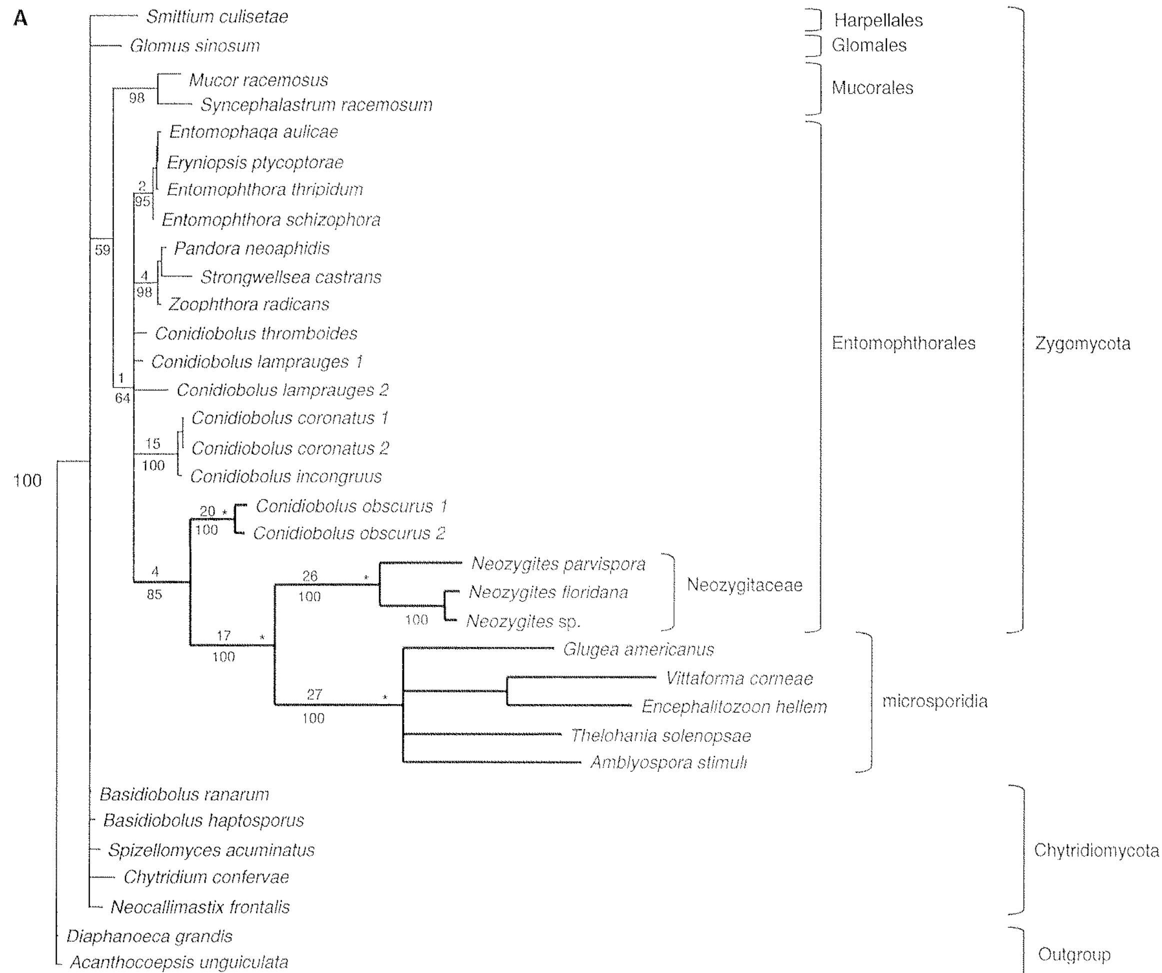
The SSU rDNA of Neozygitaceae was shorter than in other Entomophthorales:-- The SSU rDNA of three isolates of *Neozygites* species and

five other species of Entomophthorales was amplified and sequenced by PCR. It was observed that the amplified PCR product of *Neozygites* isolates was about 400 bp shorter when compared to all other Entomophthorales. However, these deletions were not included in the phylogenetic analysis because all gaps and ambiguously aligned characters were removed from the alignments. This exclusion resulted in a final alignment that consisted of 649 characters and did not change tree topology (not shown). The analysis of the lengths of randomly generated trees for all alignments used in this study was skewed to the left and contained significant phylogenetic information ($-1.00 < g_1 < -1.23$, $p<0.01$) (Hillis & Huelsenbeck, 1992).

C. obscurus*, Neozygitaceae and microsporidia formed a monophyletic branch:--* To characterise the phylogenetic relations within the order of the Entomophthorales the SSU rDNA sequences of several novel species were determined and analysed phylogenetically. Within the Entomophthorales two distinct clades were separated: one contained the genera *Entomophthora, Entomophaga* and *Eryniopsis,* and a second clade was comprised of *Pandora, Strongwellsea* and *Zoophthora* (Jensen *et al.*, 1998) (Fig. 1A). The genus *Conidiobolus* formed a paraphyletic assemblage (Fig. 1A). In our analysis we included novel sequences of *Conidiobolus* species and could thereby distinguish three groups: 1) *C. lamprauges* and *C. thromboides* were weakly resolved within the Entomophthorales, 2) *C. coronatus* and *C. incongruus* formed a distinct group strongly supported by bootstrap and decay analysis, 3) the two isolates of *C. obscurus* also formed a separate well resolved branch (Fig. 1A). Also in agreement with previous work was the relatedness of *Basidiobolus* with the phylum of the Chytridiomycota and the status of Entomophthorales and Mucorales as sister groups within the Zygomycota (Nagahama *et al.*, 1995; Jensen *et al.*, 1998) (Fig. 1A).

Most interestingly, a well resolved clade containing *C. obscurus*, Neozygitaceae and microsporidia was revealed (Fig. 1A). The association of these three groups was found identically in trees inferred by parsimony, distance or maximum likelihood methods (not shown). The clustering of *C. obscurus*, Neozygitaceae and microsporidia was supported by high bootstrap values in parsimony (85%, Fig. 1A) and distance analysis (82%, not shown). The three groups themselves formed branches with high bootstrap and decay values (100% bootstrap, decay index=20-27, Fig. 1A). Most parsimonious trees, where any of these three groups was constrained not to be monophyletic, were significantly longer as determined by rank sum tests ($p<0.005$, indicated by * in Fig. 1A). Based on both the statistical support and the high bootstrap and decay

values it was concluded that Neozygitaceae and microsporidia comprised sister groups. The consistency of the *C. obscurus*-Neozygitaceae-microsporidia branch under different tree building methods and the strong bootstrap support also confirmed the validity of this association and suggested that Neozygitaceae and microsporidia diverged from *C. obscurus*.



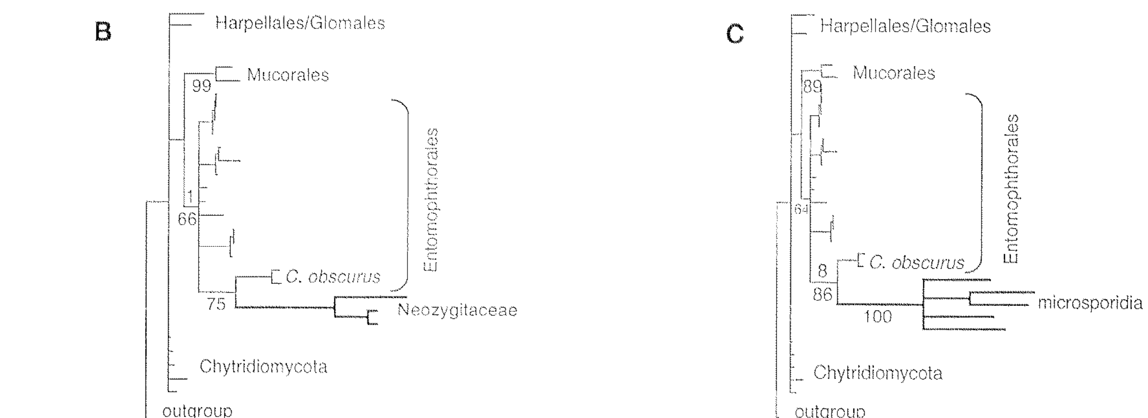


Fig. 1. Consensus trees of 1000 bootstrap replicates analysed by the parsimony method. Analysis with the full data set (A) or with data sets where microsporidian sequences (B) or *Neozygites* sequences (C) were omitted. Groups with frequencies >50% were retained. Bootstrap and decay values are indicated below and above branches respectively. Stars indicate that a tree forced not to contain the indicated branch was significantly longer.

The C. obscurus-*Neozygitaceae-microsporidia clade diverged basally from Entomophthorales:--* Parsimony, distance and likelihood analysis all detected Neozygitaceae and microsporidia reliably as sister groups within the Entomophthorales. To evaluate alternative tree topologies most parsimonious trees were searched under the constraints that either Entomophthorales and microsporidia or *C. obscurus*, Neozygitaceae and microsporidia were not monophyletic. The branch including Entomophthorales and microsporidia was found in 64% of all bootstrap replicates, but the most parsimonious tree without monophyly for this branch was only one step longer and not significantly longer than the unconstrained most parsimonious solution ($p>0.05$, Fig. 1A). If the tree search was restricted to topologies not containing *C. obscurus*, Neozygitaceae and microsporidia as a monophyletic group, the most parsimonious classification was four steps longer and not significantly longer than the unconstrained tree ($p>0.05$, Fig. 1A).

In the most parsimonious solutions under both constraints mentioned above *C. obscurus*, Neozygitaceae and microsporidia still clustered together, but formed a branch that included Mucorales, the sister group of Entomophthorales (not shown). This reinforced the designation of *C. obscurus*, Neozygitaceae and microsporidia as a separate clade additionally. The results from the constrained parsimony analysis also suggested that *C. obscurus*, Neozygitaceae and microsporidia diverged basally from Entomophthorales, close to the separation from Mucorales.

The branching pattern was identical when either Neozygitaceae or microsporidia were excluded:-- To further confirm the association of Neozygitaceae and microsporidia either one of these two groups was excluded from the analysis. If one of the two groups was present individually it branched identically from *C. obscurus* and thus confirmed the tree topology from the complete data set (Fig. 1A, B, C). The clades *C. obscurus*-Neozygitaceae (Fig. 1B) and *C. obscurus*-microsporidia (Fig. 1C) were both supported by high bootstrap values (75% and 86% bootstrap respectively, Fig. 1B, C). In addition, when *C. obscurus* was excluded Neozygitaceae and microsporidia clustered together, identical to the tree topology obtained with the complete data set (100% bootstrap, decay index=20, not shown).

Based on both the statistical support and the high bootstrap and decay values it was concluded that Neozygitaceae and microsporidia comprised sister groups. The consistency of the *C. obscurus*-Neozygitaceae-microsporidia branch under different tree building methods and the strong bootstrap support

also confirmed the validity of this association and suggested that Neozygitaceae and microsporidia diverged from *C. obscurus*.

DISCUSSION

Our phylogenetic analysis verified at the molecular level the monophyletic origin of Neozygitaceae and their belonging to the Entomophthorales as well as the fungal origin of microsporidia. In addition, it localised for the first time the phylogenetic origin of microsporidia within the fungal kingdom: microsporidia and Entomophthorales formed a monophyletic group within the Zygomycota. Within the Entomophthorales microsporidia constituted a sister group to the Neozygitaceae. In our analysis the newly determined sequences of Neozygitaceae and *C. obscurus* broke up the extremely long microsporidian branches that connected this clade to the fungal kingdom in previous analysis using ribosomal DNA sequences (Van de Peer *et al.*, 2000). This is known to improve accuracy of phylogenetic analysis (Graybeal, 1998), and the result that the branches of Neozygitaceae and microsporidia were placed identically irrespective whether they were included individually or as a group, provided the strongest support that their relationship was not an artifact (Siddall & Whiting, 1999). Therefore, the inclusion of the sequences of *Neozygites* species and *C. obscurus* rendered the phylogenetic analysis of the SSU rDNA more accurate and reliable, enabled the localisation of the microsporidian origin within the Entomophthorales, and defined the microsporidia as a sister group of the Neozygitaceae.

The classification of the microsporidia within the Entomophthorales is further supported by the observation that the microsporidian spore contains chitin (Keeling & McFaddan, 1998). The presence of chitin and glucan, as well as the absence of chitosan as a cell wall component is a specific characteristic of the Entomophthorales, absent in other orders of the Zygomycota (Bartnicki-Gracia, 1987). In addition, Neozygitaceae are characterised by nuclei with "semi-condensed" heterochromatin (Ben-Ze'ev, 1987). Similarly, microsporidian nuclei are described as containing finely dispersed chromatin (Vávra & Sprague, 1976).

Due to the fact that *Neozygites* species have been cultivated *in vitro* only recently (Grundschober *et al.*, 1998; Leite *et al.*, 2000), little is known about these fungi at a cellular or molecular level. However, there are striking similarities in the life cycles of Neozygitaceae and microsporidia. Most importantly both groups are obligate biotrophs and utilise spores as dispersing units. In microsporidia single cells divide intracellularly and develop into spores

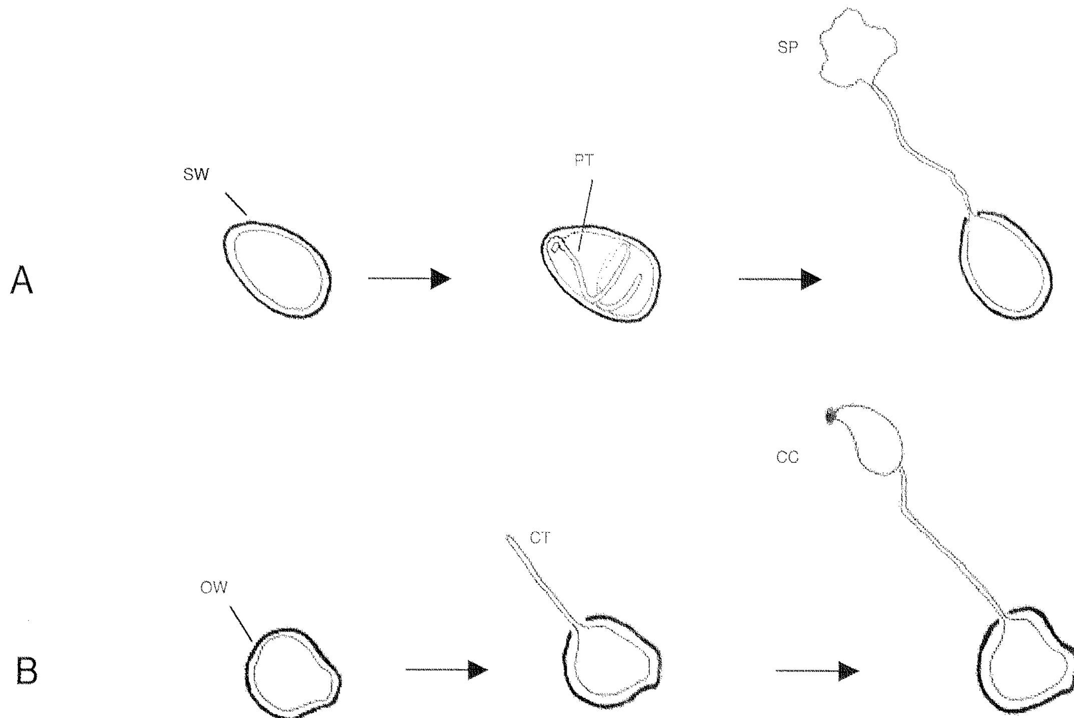


Fig. 2. A comparative model for spore germination in microsporidia (A) and secondary spore formation in Neozygitaceae (B). In microsporidia the spore, surrounded by the spore wall (SW) germinates by forming the polar tube (PT) within the spore wall. The polar tube is expelled from the spore, might penetrate a host cell and the sporoplasm (SP) is formed. In Neozygitaceae the primary spore is surrounded by an outer wall (OW), germinates and a capillary tube (CT) breaks through the outer wall. At the top of the capillary tube the secondary spore, the capilliconidium (CC), is formed.

containing a characteristic polar tube within this spore (Fig. 2A). When the spore germinates this capillary polar tube is released, penetrates the membrane of host cells and the sporoplasm is injected, starting a new infection (Vávra & Sprague, 1976) (Fig. 2A). Neozygitaceae multiply as single cells in the hemocoel of the host. Upon the switch to hyphal growth sporophores break through the cuticle of the host and single primary spores are formed within sporangia (Hesseltine & Ellis, 1973). Primary spores of Neozygitaceae therefore contain an outer cell wall originating from the sporangium and an inner spore wall (Fig. 2B). The primary spores are actively ejected, germinate and give rise to long slender capillary tubes at the top of which a capilliconidium is formed (Keller, 1997) (Fig. 2B). This capilliconidium sticks to the body of passing arthropods and the fungus penetrates the host cuticle thus starting a new infection cycle. We propose that spore germination in microsporidia represents a variation of secondary spore formation as described for Neozygitaceae. According to this hypothesis the wall of the microsporidian spore is similar to the outer cell wall of an entomophthoralean primary spore. As in Neozygitaceae a

long, thin capillary tube, the polar tube, emerges except that in microsporidia it remains enclosed within the sporangium resulting in the typical coiled polar tube visible inside matured spores (Didier *et al.*, 1998) (Fig. 2A). The cytoplasm that moves through the capillary (as in Neozygitaceae) is released as a sporoplast, analogous to the secondary spore of Neozygitaceae (Fig. 2B). This model of spore germination in microsporidia solely builds on mechanisms of spore formation observed in their phylogenetic sister group and unifies different theories about microsporidian spore germination (Dissanaike & Canning, 1957; Lom & Vávra, 1963; Canning, 1993) by allowing variation of the moment at which the polar tube is expelled from the primary spore. If the capillary is released at an early stage elongation of this structure and formation of the sporoplasm take place outside the primary spore. In contrast, the whole "capillisporoplasm" could be completed within the sporangium and be liberated as a complete unit. Our hypothesis implies predictions that can be addressed experimentally. As an example it remains to be seen whether the ultrastructure of the capillary tube in Neozygitaceae and the polar tube of microsporidia is the same.

Our results also suggest a much broader distribution and more successful strategy of entomophthoralean fungi than previously anticipated. The Entomophthorales have been taken as a unique example for the direction of an evolutionary path leading from saprobic to obligate pathogenic and extracellular parasitic organisms (Humber, 1984). Our analysis now suggests a further level in entomophthoralean evolution: intracellular parasitism as represented by the microsporidian clade. It can be speculated that the microsporidian fungi lost mitochondria in the course of their evolution to highly specialised intracellular parasites.

ACKNOWLEDGEMENTS

We would like to thank M. Soliva (Geobotanical Institute, ETH Zurich) and K. Loeffel, U. Kües and A. Grundschober for their helpful comments, discussions and suggestions. We thank R. Humber, ARS-Collection of Entomopathogenic fungi, USDA, for providing two *Conidiobolus* and one *Neozygites* isolate. This work was financed by the Kommission für Technologie und Innovation (KTI), Novartis Crop Protection AG, Basel, and the ETH Zürich.

REFERENCES

Bartnicki-Garcia, S. (1987). The cell wall: a crucial structure in fungal evolution. In *Evolutionary biology of the fungi*, pp. 389-403. Edited by Rayner, A. D. M., Brasier, C. M., & Moore, D.: Cambridge University Press.

Ben-Ze'ev, I., Kenneth, R., G. and Uziel, A. (1987). A reclassification of *Entomophthora turbinata* in *Thaxterosporium* gen. nov., Neozygitaceae fam. nov. (Zygomycetes: Entomophthorales). *Mycotaxon* **28**, 313-326.

Bruns, T. D., Vilgalys, R., Barns, S. M., Gonzalez, D., Hibbett, D. S., Lane, D. J., Simon, L., Stickel, S., Szaro, T. M. & *et al.* (1992). Evolutionary relationships within the fungi: Analyses of nuclear small subunit rRNA sequences. *Mol Phylogenet Evol* **1**, 231-241.

Butt, T. M. & Humber, R. A. (1989). An immunofluorescence study of mitosis in a mite-pathogen, *Neozygites* sp. (Zygomycetes: Entomophthorales). *Protoplasma* **151**, 115-123.

Canning, E. U. (1993). Microsporidia. In *Parasitic protozoa*, pp. 299-370. Edited by Kreier, J. P. San Diego: Academic Press, Inc.

Carl, K. P. (1975). An *Entomophthora* sp. (Entomophthorales: Entomophthoraceae) pathogenic to *Thrips* spp. (Thysan.: Thripidae) and its potential as a biological control agent in glasshouses. *Entomophaga* **20**, 381-388.

Cavalier-Smith, T. (1983). A 6-kingdom classification and a unified phylogeny. In *Endocytobiology II*, pp. 1027-1034. Edited by Schenk, H. E. A. & Schwemmler, W. Berlin: De Gruyter.

Cavalier-Smith, T. (1987). The origin of fungi and pseudofungi. In *Evolutionary Biology of the Fungi*, pp. 339-353. Edited by Rayner, A. D. M., Brasier, C. M., & Moore, D. M. Cambridge: Cambridge University Press.

Cavalier-Smith, T. (1998). A revised six-kingdom system of life. *Biol Rev* **73**, 203-266.

Delalibera, I. J., Gomez, D. R. S., Moraes, G. J. D., Alencar, J. A. D. & Araujo, W. F. (1992). Infection of *Mononychellus tanajoa* (Acari: Tetranychidae) by the fungus *Neozygites* sp. (Entomophthorales) in Northeastern Brazil. *Florida Entomologist* **75**, 145-147.

Didier, E. S., Snowden, K. F. & Shadduck, J. A. (1998). Biology of microsporidian species infecting mammals. *Adv Parasitol* **40**, 283-320.

Dissanaike, A. S. & Canning, E. U. (1957). The mode of emergence of the sporoplasm in microsporidia and its relation to the structure of the spore. *Parasitology* **47**, 92-99.

Edlind, T. D., Li, J., Visvesvara, G. S., Vodkin, M. H., McLaughlin, G. L. & Katiyar, S. K. (1996). Phylogenetic analysis of β-tubulin sequences from amitochondrial protozoa. *Mol Phylogenet Evol* **5**, 359-367.

Fast, N. M., Logsdon, J. M. J. & Doolittle, W. F. (1999). Phylogenetic analysis of the TATA box binding protein (TBP) gene from *Nosema locustae*: Evidence for a microsporidia-fungi relationship and spliceosomal intron loss. *Mol Biol Evol* **16**, 1415-1419.

Germot, A., Philippe, H. & Le Guyader, H. (1997). Evidence for loss of mitochondria in Microsporidia from a mitochondrial-type HSP70 in *Nosema locustae*. *Mol Biochem Parasitol* **87**, 159-168.

Graybeal, A. (1998). Is it better to add taxa or characters to a difficult phylogenetic problem? *Syst Biol* **47**, 9-17.

Groth, J. G. & Barrowclough, G. F. (1999). Basal divergences in birds and the phylogenetic utility of the nuclear RAG-1 gene. *Mol Phylogenet Evol* **12**, 115-123.

Grundschober, A., Tuor, U. & Aebi, M. (1998). *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *Syst Appl Microbiol* **21**, 461-469.

Hesseltine, C. W. & Ellis, J. J. (1973). Mucorales. In *The Fungi, an advanced treatise*, pp. 187-217. Edited by Ainsworth, G. C., Sparrow, F. K., & Sussman, A. S. NY: Academic Press.

Hillis, D. M. & Huelsenbeck, J. P. (1992). Signal, noise, and reliability in molecular phylogenetic analysis. *J Hered* **83**, 189-195.

Hirt, R. P., Healy, B., Vossbrinck, C. R., Canning, E. U. & Embley, T. M. (1997). A mitochondrial Hsp70 orthologue in *Vairimorpha necatrix*: molecular evidence that microsporidia once contained mitochondria. *Curr Biol* **7**, 995-998.

Hirt, R. P., Logsdon Jr., J. M., Healy, B., Dorey, M. W., Doolittle, W. F. & Embley, T. M. (1999). Microsporidia are related to fungi: evidence from the largest subunit of RNA polymerase II and other proteins. *Proc Natl Acad Sci USA* **96**, 580-585.

Hoddinott, J. & Olsen, O. A. (1972). A study of the carbohydrates in the cell walls of some species of the Entomophthorales. *Can J Bot* **50**, 1675-1679.

Humber, R. A. (1984). Foundations for an evolutionary classification of the Entomophthorales (Zygomycetes). In *Fungus-Insect Relationships: Perspectives In Ecology and Evolution*, pp. 166-183. Edited by Wheeler, Q. & Blackwell, M. New York, N.Y., USA: Columbia University Press.

Jensen, A. B., Gargas, A., Eilenberg, J. & Rosendahl, S. (1998). Relationships of the insect-pathogenic order Entomophthorales (Zygomycota, Fungi) based on the phylogenetic analysis of small ribosomal sequences (SSU rDNA). *Fungal Genet Biol* **24**, 325-334.

Kamaishi, T., Hashimoto, T., Nakamura, Y., Masuda, Y., Nakamura, F., Okamoto, K., Shimizu, M. & Hasegawa, M. (1996). Complete nucleotide sequences of the genes encoding translation elongation factors 1α and 2 from a microsporidian parasite, *Glugea plecoglossi*: implications for the deepest branching of eukaryotes. *J Biochem* **120**, 1095-1103.

Keeling, P., J. & Doolittle, W. F. (1996). Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. *Mol Biol Evol* **13**, 1297-1305.

Keeling, P. J. (2000). Evidence from beta-tubulin phylogeny that microsporidia evolved from within the fungi. *Mol Biol Evol* **17**, 23-31.

Keeling, P. J. (1998). A kingdom's progress: archezoa and the origin of eukaryotes. *BioEssays* **20**, 87-95.

Keeling, P. J. & McFaddan, G. I. (1998). Origins of microsporidia. *Trends Microbiol* **6**, 19-23.

Keller, S. (1997). The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. *Sydowia* **49**, 118-146.

Leite, L. G., Smith, L., Moraes, G. J. & Roberts, D. W. (2000). *In vitro* production of hyphal bodies of the mite pathogenic fungus *Neozygites floridana*. *Mycologia* **92**, 201-207.

Lom, J. & Vávra, J. (1963). The mode of sporoplasm extrusion in microsporidian spores. *Acta Protozool* **1**, 81-89.

Mathis, A. (2000). Microsporidia: emerging advances in understanding the basic biology of these unique organisms. *Int J Parasitol* **30**, 795-804.

Müller, M. (1997). What are the microsporidia? *Parasitol Today* **13**, 455-456.

Nagahama, T., Sato, H., Shimazu, M. & Sugiyama, J. (1995). Phylogenetic divergence of the entomophthoralean fungi: evidence from nuclear 18S ribosomal RNA gene sequences. *Mycologia* **87**, 203-209.

Peyretaillade, E., Broussolle, V., Peyret, P., Metenier, G., Gouy, M. & Vivares Christian, P. (1998). Microsporidia, amitochondrial protists, possess a 70-kDa heat shock protein gene of mitochondrial evolutionary origin. *Mol Biol Evol* **15**, 683-689.

Siddall, M. E. & Whiting, M. F. (1999). Long-branch abstractions. *Cladistics* **15**, 9-24.

Solter, L. F. & Maddox, J. V. (1998). Microsporidia as classical biological control agents: research and regulatory issues. *Phytoprotection* **79 (SUPPL.)**, 75-80.

Steinkraus, D. C., Kring, T. J. & Tugwell, N. P. (1991). *Neozygites fresenii* in *Aphis gossypii* on Cotton. *Southwest Entomol* **16**, 118-123.

Swofford, D. L. (1999). *PAUP 4.0b4a. Phylogenetic analysis using parsimony*, 4 edn. Sunderland, Massachusetts: Sinauer Associates.

Thaxter, R. (1888). The Entomophthorae of the United States. *Mem Boston Soc Nat Hist* **4**, 133-201.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignement aided by quality analysis tools. *Nucleic Acids Res* **24**, 4876-4882.

Van de Peer, Y., Ben Ali, A. & Meyer, A. (2000). Microsporidia: accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. *Gene* **246**, 1-8.

Vávra, J. & Sprague, V. (1976). Biology of the microsporidia. In *Comparative Pathobiology*, pp. 371. Edited by Bulla, L. A. J. & Cheng, T. C. New York: Plenum Press.

Vossbrinck, C. R., Maddox, J. V., Friedman, S., Debrunner-Vossbrinck, B. A. & Woese, C. R. (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature* **326**, 411-414.

Vossbrinck, C. R. & Woese, C. R. (1986). Eukaryotic ribosomes that lack a 5.8S RNA. *Nature* **320**, 287-288.

Zolan, M. E. & Pukkila, P. J. (1986). Inheritance of DNA methylation in *Coprinus cinereus*. *Mol Cell Biol* **6**, 195-200.

Seite Leer /
Blank leaf

CHAPTER 3

The MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities

Florian M. Freimoser
Claude A. Jakob[2]
Markus Aebi
Urs Tuor[1]

ETH Zurich
Mikrobiologisches Institut
Schmelzbergstr. 7
CH-8092 Zürich, Switzerland

Applied and Environmental Microbiology, 65(8), 1999, p. 3727-3729
Copyright © 1999, American Society for Microbiology.
All Rights Reserved.

Received 15 January 1999/Accepted 8 May 1999
[1] Corresponding author. Mailing address: ETH Zürich, Institute für Mikrobiologie, Schmelzbergstr. 7, CH - 8092 Zurich, Switzerland. Phone: 41/1/632 40 01. Fax: 41/1/632 11 48. E-mail: tuor@micro.biol.ethz.ch.
[2] present address: McGill University, Department for Anatomy & Cell Biology, Montreal PQ, Canada, H3A 2B2.

Seite Leer /
Blank leaf

The MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities

The entomopathogenic fungus *Neozygites parvispora* (Entomophthorales: Zygomycetes) grows *in vitro* as irregularly rod shaped hyphal bodies in a complex medium. In order to simplify the medium composition and determine growth-promoting compounds for the cultivation of this fungus we were looking for a rapid and quantitative method to estimate the number of living cells in small volumes of liquid culture. A colorimetric method for the determination of cell densities using MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) proved to be more accurate and time saving than conventional hemocytometer counts.

The entomopathogenic fungus *Neozygites parvispora* (Entomophthorales: Zygomycetes) was recently cultivated for the first time in a medium containing insect hemolymph, fetal bovine serum (FBS) and Grace's insect cell culture medium (Grundschober *et al.*, 1998). This fungus grows in liquid culture as irregularly shaped, discrete hyphal bodies. Since hemolymph is difficult to obtain and only available in limited amounts, cultivation is limited to small volumes. The identification of growth factors and the simplification of this complex medium required the determination of cell densities in many samples. We were therefore looking for an accurate and rapid method for the determination of cell densities in small culture volumes. Methods commonly used for this purpose are hemocytometer counting, determinations of protein content, wet or dry weight measurements and determination of the optical density. While hemocytometer counting and protein determinations have the disadvantage of being time consuming and tedious, the measurement of wet or even dry weights is not practicable for very small culture volumes. The measurement of the optical density works well if cell shapes are regular, as for example in yeast, but in our case it is problematic because of the irregular cell shapes and dimensions of *N. parvispora*.

An alternative method that was originally developed as a rapid assay for growth and survival of mammalian lymphoma cells is based on the transformation and colorimetric quantification of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). The respiratory chain

(Slater *et al.*, 1963) and other electron transport systems (Liu *et al.*, 1997) reduce MTT and other tetrazolium salts and thereby form water insoluble violet formazan crystals within the cell (Altman, 1976). The amount of these crystals can be quantified spectrophotometrically and serves as an estimate for the number of mitochondria and hence the number of living cells in the sample (Denizot & Lang, 1986). These features can be taken advantage of in cytotoxicity or cell proliferation assays, which are widely used in immunology, toxicology or cellular biology (Sieuwerts *et al.*, 1995).

The MTT-assay was performed as first described by Mosmann (1983) under consideration of the modifications suggested by Denizot and Lang (1986). Additionally some adjustments for the use with fungi had to be made. MTT stock solution (5 mg MTT/ml in distilled water) was filter sterilized and kept for no more than two weeks at 4°C. To start the coloring reaction, stock solution was added to growing cultures (final concentration 0.5 mg/ml). The mixture was incubated for 16 hours on a shaker (160 rpm at 20°C). Cells were pelleted in Eppendorf tubes (15000 g, 5 min), the medium was removed, 500 µl propanol were added to the cells and the tubes were vortexed. Lysed cells and debris were pelleted (15000 g, 5 min) and 100 µl of the supernatant were transferred into a 96-well ELISA plate. The optical density was measured using a spectrophotometer (SPECTRAmax PLUS, Molecular Devices) at 560 nm, with 690 nm as a reference read-out. A blank with propanol alone was measured and subtracted from all values. Measurement of a dilution series showed that the linear range extended up to OD=2.

For all experiments an isolate of *Neozygites parvispora* was grown on a shaker (160 rpm at 20°C) in 48-well microtiter plates with 500 µl medium per well. The inoculum consisted of $2.5*10^4$ cells per well. The standard liquid medium consisted of Grace's insect cell culture medium, hemolymph and FBS as described by Grundschober *et al.* (1998). In a first experiment the fungus was grown for one week to a cell density of $7*10^6$ cells/ml culture volume (cells in stationary phase) and was then incubated with MTT for different time periods. The results showed that the formazan production was saturated after 16 hours and therefore this was chosen as the incubation period for all further experiments.

The method was validated and compared with conventional hemocytometer counts. A culture of *N. parvispora* was grown and diluted with fresh medium to get cell densities between $8.45*10^6$ and $8.25*10^4$ cells/ml culture. The cell densities in the different dilutions were determined as the mean value of four hemocytometer counts (about 300 cells counted except for lowest cell densities). From each dilution five samples were measured with the MTT

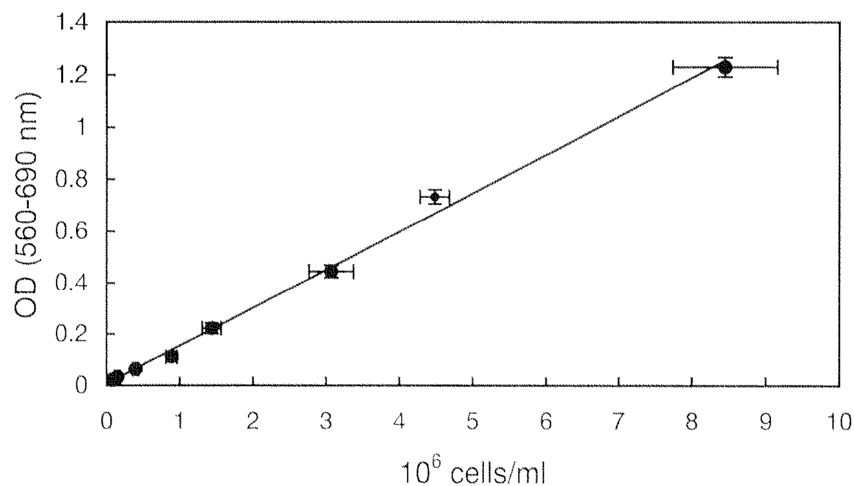


Fig. 1: Comparison of the results from the MTT method with hemocytometer counts. A dense culture of *N. parvispora* was diluted to cell densities between $8.45*10^6$ and $8.25*10^4$ cells/ml culture volume. The samples were then incubated with MTT and absorbance was measured after 16 hours. The results of both methods show a linear relationship (R^2=0.996; n=8; *P<0.0001*). Each data point represents the mean and standard error calculated from five independent MTT measurements and four hemocytometer counts.

method. The comparison of the result obtained from the two methods showed a linear relationship between the optical density measurements using MTT and the hemocytometer counts (Fig. 1). Additionally, the variability of the values determined with the MTT method was smaller.

For further evaluation of the MTT method *N. parvispora* was grown for seven days in media with variable hemolymph concentrations and different concentrations of bovine serum albumin (BSA) as a substitute for FBS. Hereby different cell densities were obtained and the MTT method could be tested under growth limiting conditions. The cell densities of four independent replicates were determined with a hemocytometer (four counts per replicate, 500-1000 cells counted except for lowest cell densities) and compared with the MTT measurement in these four replicates. The two methods again showed a linear relationship and in comparison with hemocytometer counts the MTT method was less variable (Fig. 2).

Finally, the same method was tested with another entomopathogenic fungus, *Entomophthora thripidum* (Zygomycetes: Entomophthorales). This fungus does not require hemolymph for cultivation and grows as protoplasts that form small aggregates in liquid culture (Freimoser *et al.*, 2000). To determine the cell numbers, cultures were first pipetted gently to disrupt the pellets and then the protoplasts were counted as before in a hemocytometer (four counts per sample, 200 cells counted except for lower cell densities). After a serial dilution with fresh medium the cell densities for *Entomophthora thripidum* varied between $4.75*10^5$ and $2.5*10^3$ cells/ml culture volume. For each cell density six

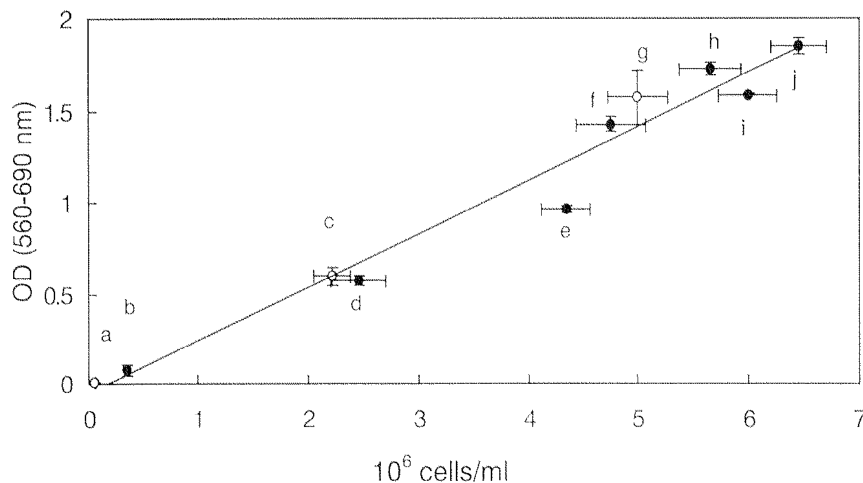


Fig. 2: Comparison between MTT assay and hemocytometer counts for *N. parvispora* grown under various media compositions in order to get different cell densities.
Grace's insect cell culture medium and FBS with increasing hemolymph concentrations (○; 0%: a; 5%: c; 10%: g). Grace's insect cell culture medium and 10% of hemolymph with different concentrations of BSA (bovine serum albumin) as a replacement for FBS (●; 0mg/ml: b; 0.625mg/ml: d; 1.25mg/ml: e; 2.5mg/ml: f; 3.75mg/ml: i; 5mg/ml: h; 10mg/ml: j). The two methods still exhibit a linear relationship (R^2=0.968; n=10; $P<0.0001$). Each data point represents the mean and standard error of four independent replicates.

samples were measured with the MTT method and the results were compared with the hemocytometer counts. As for *N. parvispora* the two measurements correlated linearly and the values from the MTT method were less variable.

The results of this study demonstrated that the transformation of the tetrazolium salt to formazan and its quantification could serve as a measure for cell densities of the two fungi *Neozygites parvispora* and *Entomophthora thripidum*. In comparison with the procedure published by Mosmann (1983) the incubation period with the MTT had to be prolonged. This was expected because these fungi grow at relatively low temperatures (20°C) compared to 37°C with vertebrate cells. Furthermore, the cell wall might act as an additional barrier for the uptake of MTT. In most other reports on the MTT method the OD measurement reached a maximum after four to eight hours and remained constant thereafter (Friedrich, 1991). However, incubation periods up to 24 hours did not have a negative influence and have been used (Kirkpatrick *et al.*, 1990). Another change to the original protocol was the removal of the medium prior to the measurement. This resulted in improvement of the accuracy and reliability (Denizot & Lang, 1986). Because all measurements were made in propanol, different complex media did not directly affect the optical densities. This was an advantage over a similar method using the tetrazolium salt XTT (sodium 3'-(1-((phenylamino)-carbonyl)-3,4-tetrazolium)-bis(4-methoxy -6-nitro) benzene-sulfonic acid hydrate) which forms a water-soluble formazan (Roehm

et al., 1991). The centrifugation and exclusion of the lysed cells from the solution before the determination of the OD was an additional step that further improved the accuracy and reliability of the results.

The advantages of the MTT procedure are the accuracy and reliability and the saving of time (Mosmann, 1983; Denizot & Lang, 1986). In our case the new method reduced the time needed for the assays by at least 80%. We also observed that the variability of the results from the optical density measurement was smaller than from the hemocytometer counts. This was not surprising since the MTT method considers all cells in a sample rather than only a small subsample. Therefore, the MTT assay resulted in more accurate and reliable estimates of cell densities than hemocytometer counts.

In conclusion the results of this study confirmed the MTT assay to be a fast, simple, cheap and accurate method (Mosmann, 1983) for the determination of cell densities of the entomopathogenic fungi tested. In particular the MTT method proved to be useful to estimate cell densities in small culture volumes and was more accurate and reliable than hemocytometer counts. The cultivation in small culture volumes and the sensitive evaluation with the MTT assay allow the screening and testing of many different substances, fractions and nutrients indispensable to the development of defined media for the cultivation of such biotrophic fungi.

Collaborative support of members of the Institute for Microbiology at Eidgenössische Technische Hochschule Zürich (ETHZ) is highly appreciated.

U. T. is thankful for financial support from the Wolfermann-Nägeli-Stiftung. This project was supported by the Kommission für Technologie und Innovation (KTI grant 3202.1), Novartis AG, Basel and the ETHZ.

REFERENCES

Altman, F. P. (1976). Tetrazolium Salts and Formazans. *Prog Histochem Cytochem* **9**, 1-56.

Denizot, F. & Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. *J Immunol Methods* **89**, 271-277.

Freimoser, F. M., Grundschober, A., Aebi, M. & Tuor, U. (2000). *In vitro* cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements and sporulation. *Mycologia* **92**, 208-215.

Friedrich, M. (1991). Zur Leistungsfähigkeit des MTT-Testes zum Nachweis von Mykotoxinen in unterschiedlichen Probenmaterialien. In *Institut für Medizinische Mikrobiologie*, pp. 234. München: Universität München.

Grundschober, A., Tuor, U. & Aebi, M. (1998). *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *System Appl Microbiol* **21**, 461-469.

Kirkpatrick, D. L., Duke, M. & Goh, T. S. (1990). Chemosensitivity testing of fresh human leukemia cells using both a dye exclusion assay and a tetrazolium dye (MTT) assay. *Leuk Res* **14**, 459-466.

Liu, Y. B., Peterson, D. A., Kimura, H. & Schubert, D. (1997). Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem* **69**, 581-593.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63.

Roehm, N. W., Rodgers, G. H., Hatfield, S. M. & Glasebrook, A. L. (1991). An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J Immunol Methods* **142**, 257-265.

Sieuwerts, A. M., Klijn, J. G. M., Peters, H. A. & Foekens, J. A. (1995). The MTT tetrazolium salt assay scrutinized: How to use this assay reliably to measure metabolic activity of cell cultures *in vitro* for the assessment of growth characteristics, IC50-values and cell survival. *Eur J Clin Chem Clin Biochem* **33**, 813-823.

Slater, T. F., Swyer, B. & Sträuli, U. (1963). Studies on succinate-tetrazolium reductase systems: III. Points of coupling of four different tetrazolium salts. *Biochim Biophys Acta* **77**, 383-393.

CHAPTER 4

Characterization of growth factors from hemolymph for the cultivation of the entomopathogenic fungus *Neozygites parvispora*

Unpublished results

Characterization of growth factors from hemolymph for the cultivation of the entomopathogenic fungus *Neozygites parvispora*

Neozygites parvispora (Zygomycota, Entomophthorales) is an obligate biotrophic fungus that attacks different species of thrips (Thysanoptera, Thripidae). Recently this fungus has been isolated and cultivated _in vitro_ for the first time. However, for the successful isolation and cultivation of _N. parvispora_ it is necessary to add insect hemolymph to the growth medium. The goal of this study was to identify the growth factors present in hemolymph that are required by this fungus. A low and a high molecular weight (LMW and HMW respectively) fraction acted synergistically as growth factors for _N. parvispora_. Whereas the HMW activity could be replaced by fetal bovine serum (FBS), the LMW was specific for hemolymph. Characterization of this LMW activity showed that it was heat stable up to 100°C, labile under acid conditions and not soluble in organic solvents and that the size was between 100 and 500 Da. These findings are the basis for the further purification and identification of this growth factor.

INTRODUCTION

Entomophthorales are common pathogens of many insect species and they regularly cause epizootics. Therefore, it has often been tried to take advantage of these fungi to control insect pests. The demanding nutrient requirements of Entomophthorales are a major restriction to their successful deployment as biological control agents. Species belonging to the genus *Neozygites* are most difficult and choosy concerning the requirements for *in vitro* cultivation and thus, many species have not been isolated yet. *Neozygites floridana* grows *in vitro* in an insect cell culture medium supplemented with FBS (Leite *et al.*, 2000). The species *N. parvispora*, that is infectious to thrips, has also been successfully cultivated (Grundschober *et al.*, 1998). This species is unique for its dependence on insect hemolymph for growth *in vitro*. A specific starvation for hemolymph on the other hand induces the rod shaped cells of *N. parvispora* to differentiate into hyphae that will eventually lead to the formation of infectious spores (Grundschober, 2000).

Such an absolute and apparently specific requirement for insect hemolymph has not been observed for any other entomopathogenic fungus. But insect blood has been commonly used as a supplement for the cultivation of insect cells *in vitro* and substances that stimulate proliferation of insect cells have been characterized (Vaughn & Louloudes, 1978; Ferkovich & Oberlander, 1991). In the rearing of parasitoid wasps, which are being used for biological control of insect pests, hemolymph was used as an additive to artificial diets or as an ovipositional kairomone (Kainoh *et al.*, 1982; Heath *et al.*, 1990; Guerra & Robacker, 1991; Hu *et al.*, 1998). Often the identification of active substances is hampered by difficult, time consuming and inefficient assays and by the complexity of insect hemolymph as such. Consequently, the knowledge about biologically active compounds from insect blood is sparse, whereas reports on general properties and composition are abundant. Insect hemolymph is especially noteworthy for its exceptionally high content of magnesium, proteins, free amino acids, organic acids, phosphates and certain sugars (Wyatt, 1961; Wyatt, 1967; Jeuniaux, 1971; Mullins, 1985). In contrast to the rearing of endoparasitic insects, hemolymph was soon abandoned in insect cell culture and replaced by FBS (Schlaeger, 1996).

In this report it is shown that for *N. parvispora* it was not possible to replace hemolymph with FBS. Instead, hemolymph contained a high and a low molecular weight (HMW and LMW respectively) component that acted synergistically in enabling growth of *N. parvispora*. It was possible to replace the HMW fraction with FBS, whereas the LMW activity was specific for hemolymph and could not be replaced. These results and the characterization of the growth promoting activities represent the first steps for the purification and finally the identification of the growth factors that are necessary for the *in vitro* cultivation of *N. parvispora*.

MATERIAL AND METHODS

Cultivation and growth assay:-- All experiments were performed with the isolate 301 of *N. parvispora* (ARSEF 5620). The fungus was initially cultivated in a medium based on Grace's insect cell culture medium 2x supplemented with lactalbumin hydrolysate and yeastolate (Gibco No. 11667, for exact composition see appendix A). It was diluted to 1x by adding 20% heat inactivated FBS (Gibco), 10% hemolymph from *Manduca sexta* and 20% water. This medium was simplified by using unsupplemented Grace's medium 1x (Gibco No. 21590) that was diluted to 0.5x. In addition FBS was replaced with 5mg x ml^{-1} (final concentration) bovine serum albumin (BSA, Sigma, fraction V; filter sterilized).

To this medium 10% hemolymph or fractionated hemolymph samples of the corresponding concentration were added. The fractions were filter sterilized before adding to the medium (Sartorius Minisart RC 15 filters, 0.2μm). The fungus was continuously grown in 24-well microtiter plates with 1 ml of culture medium per well. The cells were subcultured twice per week when grown in the complex medium and only once per week if the fungus was cultivated in the simplified medium. Growth assays were performed in 48-well microtiter plates in 500 μl of culture medium. All cultures were kept in the dark at 20°C and shaken at 160 rpm. For assays two replicates of all media were inoculated with 10^5 hyphal bodies x ml^{-1}. All experiments were performed twice.

Collection of hemolymph -- Hemolymph was taken from 5th instar caterpillars of *Manduca sexta* by cutting one pseudoleg. The hemolymph was collected in a sterile tube on ice in order to avoid melanization. The content of each tube was heated to 65°C, centrifuged at 3000 x g for 20 min and the supernatants pooled, aliquoted and stored at -20°C until use. Caterpillars were reared by Novartis Crop Protection AG (Basel, Switzerland) and the eggs were obtained from Hoechst Schering AgrEvo GmbH (Düsseldorf, Germany) or Carolina Biological Supply Co. (Burlington, North Carolina, USA).

Evaluation of cell density: -- The cell density was assessed after one week by the MTT assay as described by Freimoser *et al.* (1999) (see chapter 3).

Characterization and fractionation of growth promoting activities in hemolymph:-- The high and low molecular weight components of hemolymph were separated on a Sephadex G-25 desalting column (Pharmacia PD-10 desalting column, exclusion limit 5 kDa), which was loaded with 1 ml of hemolymph and eluted with 9 ml of distilled water. The first 5 ml of eluent formed the HMW pool and the rest the LMW fraction.

- The heat stability of the growth factors was assessed by heating 1 ml of hemolymph to 80°C and to 100°C for 10 min and by autoclaving at 121°C and 1 bar for 20 min. The samples were centrifuged for 10 min at 10'000 x g and the supernatant tested for the presence of growth activity.

- The solubility of the LMW growth factor in the following organic solvents was tested: Methanol (99%), Ethanol (98%), Chloroform, Acetonitril, Ethyl acetate (EtOAc), DMSO (dimethylsulfoxide), Trifluoracetic acid (0.1%) and ortho-phosphoric acid (1%). The experiment was performed by freeze-drying 500 μl of heated hemolymph (100°C) and by adding the equal solvent volume. The

insoluble part was pelleted and the soluble fraction in the supernatant was removed. The separated soluble and the insoluble fractions were then freeze-dried again. After resuspension in water and filter sterilization the individual soluble or insoluble fractions as well as a mixture of both were tested. Hemolymph was also brought to acidic pH with either HCl or acetic acid or to basic pH by using NaOH. The solubility in organic solvents was tested for acidic and basic hemolymph as well. For the assay these fractions were neutralized again.

• Pretreated hemolymph (1.5 ml, extracted with $CHCl_3$, heated to 100°C, filtered through 5 kDa cut off membrane) was incubated with Chelex™ 100 (0.2 g, Bio-Rad Laboratories) that was washed three times with water before. The mixture was kept at room temperature for 2 hours. The resin was then pelleted and the supernatant tested for presence or absence of growth promoting activity.

• Size fractionation: The size of the LMW activity was determined by filtering through membranes with pore sizes of 5, 10, and 30 kDa. In addition, 500 µl of hemolymph were dialyzed in membranes of 0.1 kDa, 0.5 kDa, 1 kDa, 2 kDa, 3.5 kDa and 5 kDa cut off (Spectra/Por® DispoDialyzers®, Spectrum®) against water for 16 hours.

• Solid phase extraction: The affinity towards various resins was studied by passing 4 ml of hemolymph (10-fold diluted) through the following solid phase extraction (SPE) cartridges: reversed phase (RP) resin C18, RP C8, RP C2, RP CH, strong anion (SAX) and cation (SCX) exchange material (Extract-Clean™ SPE Tubes, 500 mg, Alltech). RP cartridges were washed with 5 ml Methanol and rinsed with 5 ml water. After application of the hemolymph sample tubes were washed with water and eluted with chloroform ($CHCl_3$). SAX and SCX cartridges were washed with 10 ml water before the application of the hemolymph sample. The tubes were then washed with 3 ml water and eluted with 5 ml ammonium bicarbonate (NH_4HCO_3, between 10 mM and 400 mM). All eluents were freeze dried, redissolved in 1 ml of distilled water and added to the culture media.

• Protease treatment: Pretreated hemolymph (200 µl, heated to 100°C, extracted with $CHCl_3$, filtered through 5 kDa cut off membrane) was incubated at 37°C for 4 h (protease K, protease from *S. griseus*) or 16 h (carboxypeptidases A and Y) with the following enzyme concentrations: protease K (0.3 U, 0.6 U, 1.2 U), protease from *S. griseus* (6.4 U), carboxypeptidase Y (1 U, 5 U, 50 U), carboxypeptidase A (1.1 U, 5.5 U, 55.5 U). For controls, hemolymph samples

were incubated with water and with proteases that had been heated to 100 °C previously. After digestion enzymes were removed by passing the samples through 3 kDa cut off filters.

- Separation on Sephadex column: Hemolymph was fractionated on a Sephadex G-25 (fine, Pharmacia) column (∅ 7 cm, length 78 cm). This column was loaded with 85 ml hemolymph (pretreated by heating to 100°C and extraction with $CHCl_3$). It was eluted with distilled, degassed water. The flow rate was approx. 2.5 ml x min^{-1} and the fraction collector was set to 10 min x $fraction^{-1}$. A chromatogram was recorded by detection of the absorbance at 206 nm using a Pharmacia LKB Uvicord SII detector. The whole run took 28 hours and yielded 168 fractions. These fractions were combined to 8 pools according to the chromatogram (Fig. 3, Tab. 1). The pooled fractions were frozen and freeze-dried. The tara of the flasks was determined and the dry weight in each pool was recorded. The dry material in the eight pools was resuspended in water and tested for growth promoting activity.

- Separation on a Dowex 50W cation exchange column: The active pool from the fractionation on the Sephadex column was further fractionated on a Dowex® 50W X8 20-50 mesh (swollen) column (∅ 1.6 cm, 60 cm, ~110 ml) in the NH_4^+-form. The column was loaded with 2-3 ml of hemolymph sample (pool 5 from Sephadex column). It was eluted with distilled, degassed water at a flow rate of approx. 0.625 ml x min^{-1}. The fraction collector was set to 4 min x $fraction^{-1}$. The whole run took 5 hours and resulted in 76 fractions. Of each fraction 100 μl were transferred to a quartz 96-well microtiter plate and absorption was measured in a spectrophotometer (SPECTRAmax Plus, Molecular Devices) at 200 nm, 230 nm and 270 nm. Based on the chromatogram the fractions were again put together to eight pools (Fig. 4, Tab. 2). The pooled fractions were frozen, freeze dried and the dry weight determined as before. The dry material in the 8 pools of the first run was again resuspended in distilled water and tested for growth promoting activity.

Altogether 7 runs were performed on this column to separate all of the active pool from the Sephadex column. The fractions of all runs that corresponded to those from the first run containing growth promoting activity were pooled, freeze dried and given to Dr. H. Ruegger (Inorganic Chemistry, ETH Zurich) for NMR analysis.

RESULTS

The course of action pursued for the identification of the growth promoting substances from hemolymph required by *N. parvispora* was a step-wise procedure. First, chemical characteristics were determined by extractions and crude size fractionations. Based on these results methods for column fractionation were chosen. Compounds in the fractions containing growth promoting activity were then identified by NMR analysis. Besides this strategical proceeding several compounds are known to occur in insect hemolymph or that play an important role in insect physiology were tested (listed in Appendix B).

Separation of high and low molecular weight components: -- Hemolymph was separated into a HMW and a LMW fraction using a Sephadex G-25 desalting column. Neither the HMW nor the LMW fraction supported growth comparable to a culture with 10% hemolymph (standard, Fig. 1). By combining both the HMW and the LMW in equal concentrations the fungus reached almost the same cell density as in the positive control (Fig. 1).

Heating hemolymph to 100°C destroyed the growth promoting activity and a medium containing FBS alone as a substitute did not allow *N. parvispora* to grow either (Fig. 1). Mixing the LMW fraction and hemolymph previously heated to 100°C led to poor growth and the effect seemed additive (Fig. 1). Combination of the HMW fraction with hemolymph heated to 100°C or heated

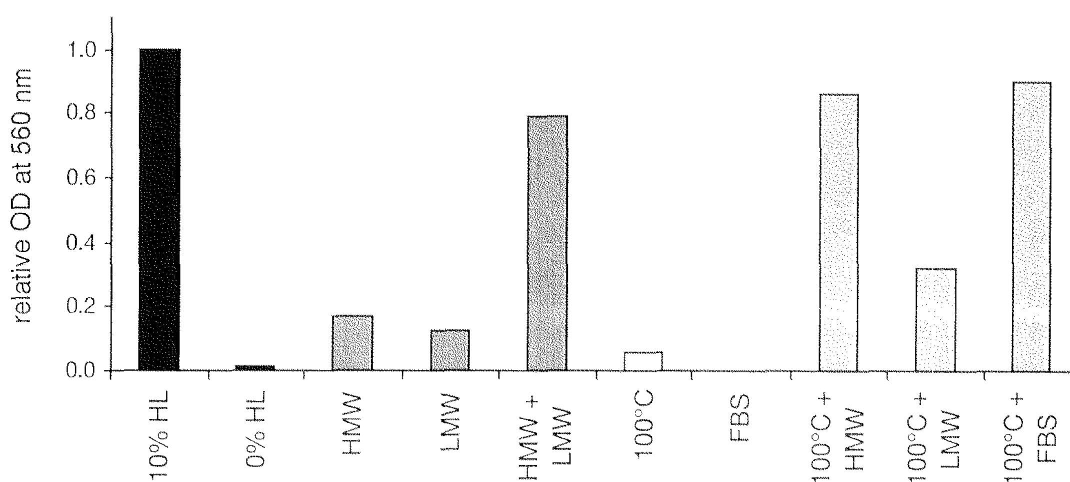


Fig. 1: Growth of *N. parvispora* in media containing different hemolymph fractions as measured by optical density at 560 nm with the MTT assay (Freimoser et al. 1999). As control the fungus was grown with 10% hemolymph (black, defined as relative OD=1) and without hemolymph (black, defined as relative OD=0). In either the HMW or the LMW pool little growth was observed. *N. parvispora* grew if both fractions were combined. Neither in hemolymph heated to 100°C nor in FBS alone did *N. parvispora* grow. Combination of the heated hemolymph with either the HMW fraction or with FBS allowed the fungus to grow. A mixture of the boiled hemolymph with the LMW fraction supported only poor growth.

hemolymph with FBS in contrast enabled *N. parvispora* to grow comparable to the positive control containing 10% hemolymph (Fig. 1). In contrast to heating to 100°C, autoclaving destroyed all growth enhancing effect of hemolymph (not shown).

Solubility of LMW growth promoting activity: -- It was tried to extract the growth promoting activity from freeze-dried hemolymph with a number of organic solvents. Except for methanol, where little activity could be dissolved, the growth promoting activity was insoluble in all solvents tested (Fig. 2). It was further tried to extract the growth factor(s) at either acidic or basic pH. In both cases the growth promoting activity was abolished (not shown). After treatment with phosphoric acid (0.1 %) no growth enhancing effect of hemolymph could be detected either (Fig. 2).

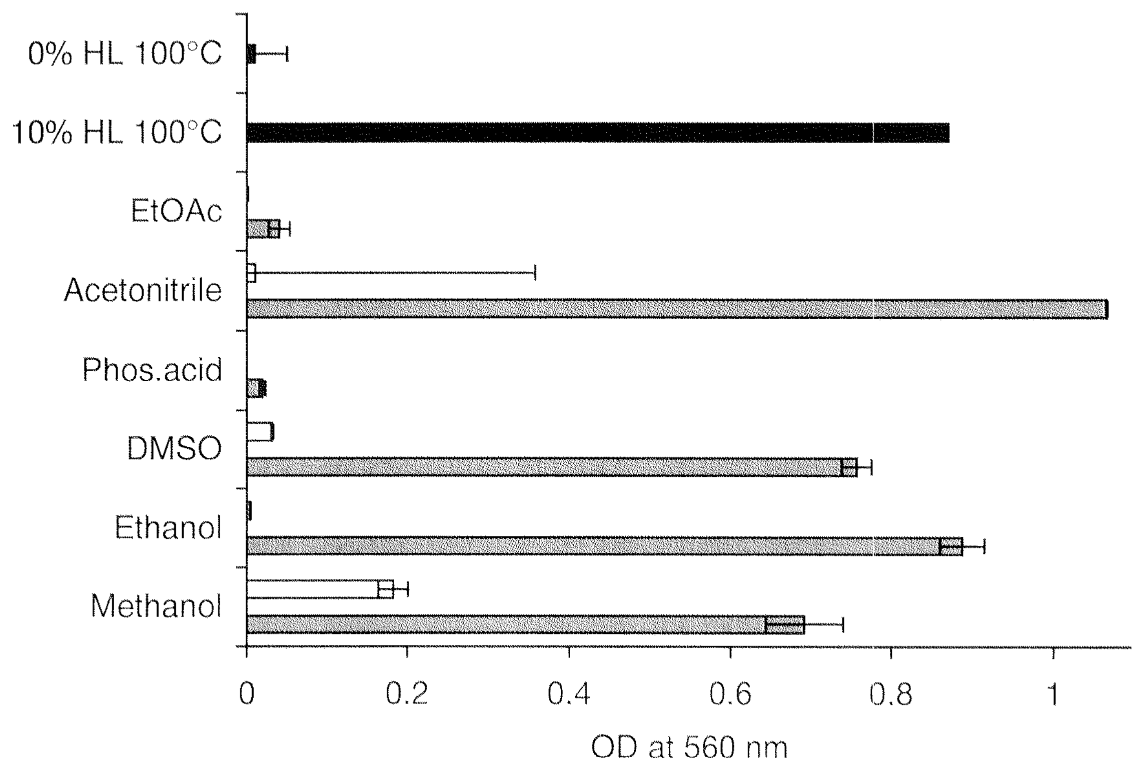


Fig. 2: Effect of treatment with diluted acid and extraction with organic solvents on the growth promoting activity from freeze-dried hemolymph. Growth was measured with the MTT assay and is expressed as optical density at 560 nm. As controls served cultures grown with 10% heated hemolymph or without hemolymph (black). Grey bars: Growth with the insoluble material. White bars: Growth with the material soluble in the corresponding solvent. EtOAc stands for ethyl acetate. DMSO stands for dimethylsulfoxide. Bars represent standard errors.

Further characterization of LMW growth factor(s): -- Dialysis with membranes of different pore sizes was performed. The majority of the growth enhancing activity passed through a 500 Da membrane, but was retained by a pore size of 100 Da. Neither treatment with chelex or with proteases affected the growth

promoting activity present in hemolymph. The growth factor(s) did not bind to any reverse phase material or to anion exchange resin. A strong cation exchanger in the H^+-form bound the growth promoting activity. It was possible to elute an active fraction with 200 mM NH_4HCO_3. In the NH_4^+-form the cation exchanger did not bind the growth promoting activity anymore.

Separation on Sephadex column: -- Fractionation of 85 ml of pretreated hemolymph on a Sephadex G-25 column and detection at 206 nm resulted in a chromatogram that exhibited 8 peaks (Fig. 3). The fractions contributing to these 8 peaks were pooled, freeze dried and resuspended in differing volumes of water, depending on the solubility (Tab. 1). Only pools 3, 5 and 6 allowed measurable growth and relative growth activity was highest in pool 5. Therefore, pool 5 was taken for further separation on a Dowex cation exchange resin.

Separation with cation exchange column: -- Batch experiments and results from

Pool	Fractions	Dry weight [g]	Growth activity [OD/mg]
1	1-33	0.01	-
2	34-51	0.07	-
3	52-60	0.04	0.01
4	61-77	1.76	-
5	78-93	1.87	0.04
6	94-115	0.58	0.02
7	116-140	0.06	-
8	141-168	0.00	-

Tab. 1: The 8 pools from the fractionation of 85 ml of hemolymph on the Sephadex G-25 column with the pooled fractions, the dry weight and the growth activity.

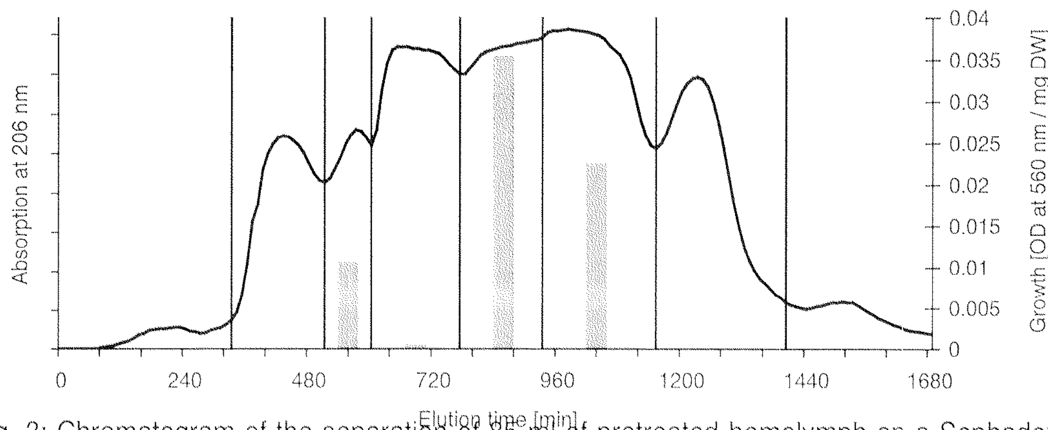


Fig. 3: Chromatogram of the separation of 85 ml of pretreated hemolymph on a Sephadex G-25 column measured at 206 nm. The vertical lines indicate the pools that were created from the fractions. Growth of *N. parvispora* in these pools is shown by the grey bars. It is expressed as the ratio of optical density at 560 nm against the dry weight in the corresponding pool.

solid phase extraction cartridges had shown that the growth promoting activity from hemolymph bound to the Dowex cation exchange resin in the H^+-form (not shown). In the H^+-form the resin cannot be eluted with NH_4HCO_3 (due to the

formation of CO_2, disrupting the column bed). The resin was therefore brought to the NH_4^+-form which did not bind the growth promoting activity anymore (not shown). For this reason the cation exchange column was only eluted with water and the separation observed was rather due to size fractionation than to an ion exchange mechanism (The Dow Chemical Company, 1964). Nevertheless, except for the first peak, which eluted with the void volume of the column, material was retained by the resin. Based on the chromatogram of the separation on Dowex strong cation exchange resin detected at 200 nm the fractions were separated into 8 pools (Fig. 4, Tab. 2). Only the pools 3, 4 and 5 enabled *N. parvispora* to grow (Fig. 4). The relative growth activity was highest in pool 3.

Pool	Fractions	Dry weight [mg]	Growth activity [OD/mg]
1	1-12	4.7	-
2	13-20	31.8	-
3	21-24	7	44.23
4	25-29	58	12.76
5	30-34	35.2	6.53
6	35-42	76.8	-
7	43-56	62.5	0.05
8	57-76	15.1	-

Tab. 2: Dry weight and fractions that were put together for the 8 pools after fractionation on the Dowex cation exchange column.

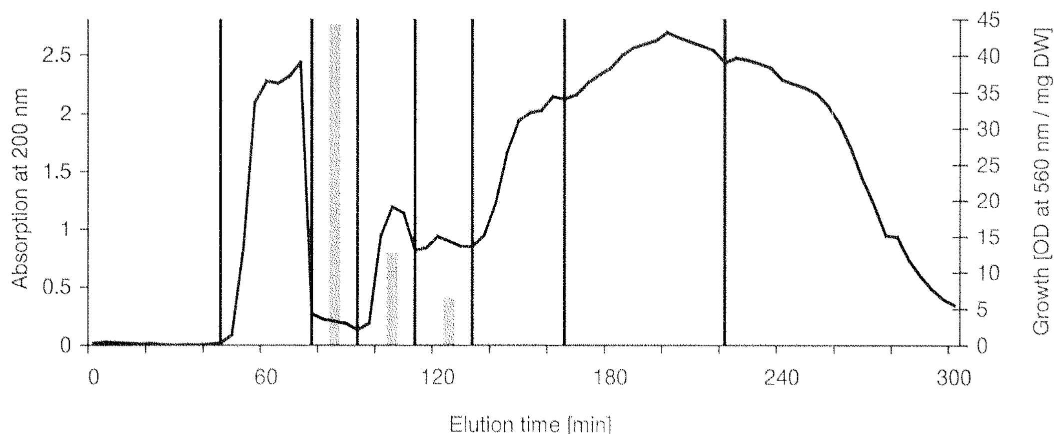


Fig. 4: Chromatogram of the separation of 2 ml of pretreated hemolymph on a Dowex strong cation exchange column measured at 200 nm. The vertical lines indicate the pools that were created from the fractions. Growth of *N. parvispora* in these pools is shown by the grey bars. It is expressed as the ratio of optical density at 560 nm against the dry weight in the corresponding pool.

NMR analysis: -- ^1H and ^{13}C NMR of active fractions from preliminary separations on Dowex cation exchange resin identified the following substances: trehalose (major component), glutamine, threonine, serine, alanine, valine and asparagine. None of these substances exhibited growth promoting activity for *N. parvispora* individually and combinations were not active either. After the fractionation by the two columns much less material was detected in

the active pool and NMR analysis detected the same substances that were previously found.

DISCUSSION

The rich medium originally used for the isolation and cultivation of *N. parvispora* contained not only insect hemolymph, but also FBS, yeastolate and lactalbumin hydrolysate (Grundschober *et al.*, 1998). It was therefore necessary to first simplify this medium so that hemolymph was the only undefined constituent. This was achieved by omitting yeastolate and lactalbumin hydrolysate and by replacing FBS with 5 mg x ml^{-1} BSA. In this medium *Neozygites parvispora* grew slower but it could be continuously cultivated if hemolymph was added.

The employment of this simplified medium made possible the discovery that hemolymph contained a HMW and a LMW fraction, which were both required for growth of *N. parvispora.* The HMW activity was destroyed by boiling and was not specific for hemolymph because it could be replaced with FBS. This finding was similar to insect cell culture where hemolymph could also be replaced by FBS (Schlaeger, 1996). The growth activity in the LMW fraction in contrast resisted to boiling, could not be replaced (see Appendix B for a list of substances tested as substitutes for the LMW fraction of hemolymph) and seemed specific for hemolymph. Therefore, characterization and purification of the LMW growth factor(s) was started. It was thus found that this activity was only soluble in water, was inactivated by acidic pH, had a molecular weight between 100 and 500 Da and was resistant to treatment with proteases. The fractionation of 85 ml of hemolymph on a Sephadex G-25 and a Dowex 50W X8 column resulted in 10.3 mg of purified active material. In this active fraction trehalose and several free amino acids were identified by NMR. However, neither trehalose nor the amino acids, which had already been present in Grace's medium (see Appendix A), served as growth factors for *N. parvispora* if tested separately or in combination. This lead to the conclusion that the growth promoting activity was present in concentrations too low for detection.

There were various properties of the presumed growth factor that complicated purification. For chromatographic separations it is favorable if samples can be applied in organic solvents and eluted either with solvents or buffers. Unfortunately, the LMW growth factor from hemolymph was insoluble in organic solvents. The use of buffers was also restricted due to the small size of the active components that made removal of salt from elution buffers impossible. Therefore, only volatile buffers could be considered for elution. For fractionation on a cation exchange resin this required the use of NH_4HCO_3 as eluent and the

column had to be used in the ammonium form. This had the effect that the growth promoting activity did not bind to the resin anymore. Another complicating issue was that hemolymph contains exceptional large amounts of molecules of similar size as the growth promoting activity.

Nevertheless, there are several possibilities that could be pursued. Probably the most obvious approach would be to screen known substances for their ability to enable *N. parvispora* to grow. During this research substances known for their occurrence in insect hemolymph have already been tested (see Appendix B). In addition, conditioned media from insect cell lines of *Manduca sexta*, extracts of plants and other complex mixtures have been tried as substitutes for hemolymph (Appendix B). In none of these cases did *N. parvispora* grow comparably to cultures in media containing hemolymph (not shown). Solely with the addition of a water extract of a commercial vitamin pill (*equate*, Vita Health Company, Winnipeg, Canada) some growth could be observed (not shown). However, with this vitamin solution *N. parvispora* grew only slowly, reached lower cell densities than with hemolymph and the effect of the vitamins was not concentration dependent. During all this time it was also never observed that *N. parvispora* adapted to cultivation and became less dependent on hemolymph, even if the isolate was continuously cultured for several months. Nevertheless, a possible approach could also be to look for isolates that do not need hemolymph or even to create mutants lacking this requirement. In my opinion the most promising continuation of this project would be to start with bigger amounts of starting material for the purification of the growth factor(s). For the isolation and purification of other growth factors or hormones much more starting material was used. The insect hormone ecdysone for example was purified from 500 kg of pupae of *Bombyx mori,* which resulted in 25 mg crystallized hormone (Butenandt & Karlson, 1954). In this case the whole insect was squeezed. This could also be tried for the isolation of the growth factor for *N. parvispora* and could replace the tedious extraction of hemolymph. However, the processing of such huge amounts of pupae or in our case caterpillars also requires special facilities and equipment enabling handling of hundreds of liters of raw extract.

The results presented here have shown that insect hemolymph contained two growth promoting fractions, both of which were mandatory for growth of *N. parvispora*. The partial characterization of the hemolymph specific, LMW growth promoting activity provides the basis for possible future attempts to purify and identify the growth factor(s) necessary for the cultivation of *N. parvispora*. During this study trehalose and several amino acids were identified in an active fraction but none of these served as growth factors for

N. parvispora. It was concluded that the inability to identify the growth promoting substance(s) was due to the limited amount of hemolymph that was used. However, I am convinced that the approach described is principally correct and suitable for the purification and identification of growth factors for *N. parvispora*.

ACKNOWLEDGEMENTS

I would like to thank Peter Wyss, Hugo Bertschy, Louis-Pierre Molleyres and Markus Müller from Novartis Crop Protection AG for various help, support and provision of material and equipment. Hoechst Schering AgrEvo GmbH (Düsseldorf, Germany) provided eggs of *Manduca sexta*. Dr. H. Ruegger (Institute of Inorganic Chemistry, ETH) carried out NMR analysis of hemolymph fractions. Rob Boulianne, Claude Jakob, Stephan TeHeesen, Helge Sierotzki and past and present members of our group have been helpful with ideas and suggestions during this research.

REFERENCES

Butenandt, A. & Karlson, P. (1954). Ueber die Isolierung eines Metamorphose-Hormons der Insekten in kristallisierter Form. *Zeitschr Naturforschung* **9b**, 389-391.

The Dow Chemical Company (1964). *Dowex ion exchange*. Midland, MI: Dow Chemical Company.

Ferkovich, S. M. & Oberlander, H. (1991). Growth factors in invertebrate *in vitro* culture. *In Vitro Cell Dev Biol* **27A**, 483-486.

Freimoser, F. M., Jacob, C., Tuor, U. & Aebi, M. (1999). The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay is a fast and reliable method for the colorimetric determination of fungal cell densities. *Appl Environ Microbiol* **65**, 3727-3729.

Grundschober, A. (2000). *In vitro* life cycle of *Neozygites parvispora* and *Entomophthora thripidum*, two fungal pathogens of *Thrips tabaci*. In *Institute of Microbiology*, pp. 127. Zurich: ETH Zurich.

Grundschober, A., Tuor, U. & Aebi, M. (1998). *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *Syst Appl Microbiol* **21**, 461-469.

Guerra, A. A. & Robacker, K. M. (1991). Potential larval diet devoid of hemolymph for *in vitro* rearing of *Bracon mellitor*. In *Proceedings Beltwide Cotton Conferences*, pp. 723. Edited by Herber, D. J. Tennessee: National Cotton council of America.

Heath, R. R., Ferkovich, S. M., Greany, P. D., Eller, F. J., Dueben, B. D. & Tilden, R. L. (1990). Progress in the isolation and characterization of a host hemolymph ovipositional kairomone for the endoparasitoid *Microplitis croceipes*. *Arch Insect Biochem Physiol* **13**, 255-265.

Hu, J. S., Gelman, D. B., Bell, R. A. & Loeb, M. J. (1998). *In vitro* rearing of *Edovum puttleri*, an egg parasitoid of the Colorado potato beetle - development from egg through the pupal stage. *BioControl* **43**, 1-16.

Jeuniaux, C. (1971). Hemolymph-Arthropoda. In *Chemical Zoology*, pp. 63-118. Edited by Florkin, H. & Sheer, B. T. New York: Academic Press.

Kainoh, Y., Hiyori, T. & Tamaki, Y. (1982). Kairomone of the egg-larval parasitoid, *Ascogaster reticulatus* (Hymenoptera: Braconidae). *Appl Entomol Zool* **17**, 102-110.

Leite, L. G., Smith, L., Moraes, G. J. & Roberts, D. W. (2000). *In vitro* production of hyphal bodies of the mite pathogenic fungus *Neozygites floridana*. *Mycologia* **92**.

Mullins, D. E. (1985). Chemistry and physiology of the hemolymph. In *Integument, Respiration and Circulation*, pp. 355-400. Edited by Kerkut, G. A. & Gilbert, L. I.: Pergamon Press.

Schlaeger, E.-J. (1996). Medium design for insect cell culture. *Cytotechnology* **20**, 57-70.

Vaughn, J. L. & Louloudes, S. J. (1978). Isolation of two growth promoting fractions from insect hemolymph. *In Vitro* **14**, 351.

Wyatt, G. R. (1961). The biochemistry of insect hemolymph. In *Annual Review of Entomology*, pp. 75-102. Edited by Steinhaus, E. A. & Smith, R. F. Palo Alto, California: Annual Reviews, Inc.

Wyatt, G. R. (1967). The Biochemistry of sugars and polysaccharides in insects. In *Advances in Insect Physiology*, pp. 287-360. Edited by Beament, J. W. L., Treherne, J. E., & Wigglesworth, V. B. London and New York: Academic Press.

APPENDIX A: Composition of Grace's insect cell culture medium.

Substance	g/l	mM	Substance	g/l	μM
Inorganic salts			**Vitamins**		
$CaCl2*2H_2O$	0.993	6.75	Para-aminobenzoic acid	2 x10^{-5}	0.146
KCl	4.1	54.99			
$MgCl2*6H_2O$	2.28	11.21	D-Biotin	1 x10^{-5}	0.041
$MgSO4*7H_2O$	2.78	11.28	D-Calcium Pantothenate	2 x10^{-5}	0.042
$NaHCO_3$	0.35	4.17	Choline Chloride	2 x10^{-3}	14.638
$NaH_2PO_4*2H_2O$	1.15	7.37	Folic acid	2 x10^{-5}	0.045
Other components			I-Inositol	2 x10^{-5}	0.111
Fructose	0.4	2.22	Niacin	2 x10^{-5}	
Fumaric acid	0.055	0.47	Nicotinic acid	2 x10^{-5}	0.162
D-glucose	0.7	3.89	Pyridoxine, HCl (B6)	2 x10^{-5}	0.097
α-Ketoglutaric Acid	0.37	2.53	Riboflavin	2 x10^{-5}	0.053
Malic acid	0.67	5.00	Thiamine, HCl	2 x10^{-5}	0.059
Succinic acid	0.06	0.51			
Sucrose	26.68	77.94			
Amino acids					
L-Alanine	0.225	2.53			
b-Alanine	0.2	2.24			
L-Arginine*HCl	0.7	3.32			
L-Asparagine	0.35	2.65			
L-Aspartic acid	0.35	2.63			
L-Cystine	0.022	0.09			
L-Glutamic acid	0.6	4.08			
L-Glutamine	0.6	4.11			
Glycine	0.65	8.66			
$L\text{-}Histidine\ HCl*H_2O$	3.335	15.91			
L-Isoleucine	0.05	0.38			
L-Leucine	0.075	0.57			
L-Lysin*HCl	0.625	3.42			
L-Methionine	0.05	0.34			
L-Phenylalanine	0.15	0.91			
L-Proline	0.35	3.04			
DL-Serine	1.1	10.47			
L-Threonine	0.175	1.47			
L-Thryptophan	0.1	0.49			
L-Tyrosine	0.05	0.28			
L-Valine	0.1	0.85			

APPENDIX B: Substances or extracts tested for their capability to replace the LMW growth promoting activity of hemolymph. None of these substances could replace the growth promoting acitvity of hemolymph in the indicated concentrations.

Substance	Concentrations
HMW components/substitutes:	
Insulin	5, 25, 50 μg x ml^{-1}
Transferrin	0.5-100 μg/ml
FBS	0.01 – 40 %
Concanavalin A	5, 20, 50 μg x ml^{-1}
Defined Lipid concentrate	0.1x, 1x, 10x
Complex Lipid concentrate	0.1x, 1x, 10x
Oleic acid (bound to BSA)	<14 nmol x ml^{-1}
BSA	0.5 – 20 mg x ml^{-1}
α-Cyclodextrin	1, 2.5, 5, 10 mg x ml^{-1}
β- Cyclodextrin	1, 2, 3, 5, 10 mg x ml^{-1}
LMW substances/substitutes:	
Amino acids:	
L-Tyrosine	0.4-120 μg x ml^{-1}
DL-Phosphoserine 0.01 M	10, 100, 300 μl x ml^{-1}
Allantoin 0.01 M	10, 100, 300 μl x ml^{-1}
Ethanolamine 0.01 M	10, 100, 300 μl x ml^{-1}
Arginine	0.5, 1 mg x ml^{-1}
Histidine	0.5, 1 mg x ml^{-1}
Lysine	0.5, 1 mg x ml^{-1}
Trehalose	0.05 - 15 mg x ml^{-1}
Putrescine	0.05 -10 μg x ml^{-1}
Spermine	0.1, 1, 10 μg x ml^{-1}
Spermidine	0.1, 1, 10 μg x ml^{-1}
L-Dopa	1-200 μg x ml^{-1}

Substance	Concentrations
Salts:	
Ammonium ferric citrate, green	12, 60, 300 mg x ml^{-1}
$CrCl_3 \cdot 6H_2O$	0.05, 0.5, 5 µg x ml^{-1}
Cu_2O	0.004, 0.04, 0.4 mg x ml^{-1}
Fe(III) Citrate (0.1 M) + EDTA (0.5 M)	0.5 – 100 µl x ml^{-1}
$FeCl_3$	0.06, 0.15, 1.5 mg x ml^{-1}
$FeSO_4$	12, 60, 300 mg x ml^{-1}
JK	0.3, 3, 30 µg x ml^{-1}
$MnCl_2$	8, 40, 200 mg x ml^{-1}
$MoNa_2O_4 \cdot 2H_2O$	0.05, 0.5, 5 µg x ml^{-1}
$NaSi_3O_7 \cdot 5H_2O$	0.02, 0.2, 2 µg x ml^{-1}
$NaVO_3 \cdot H_2O$	0.02, 0.2, 2 µg x ml^{-1}
$NiSO_4 \cdot 7H_2O$	0.01, 0.1, 1 µg x ml^{-1}
$SnCl_2 \cdot 2H_2O$	0.02, 0.2, 2 µg x ml^{-1}
ZnO	0.03, 0.3, 3 mg x ml^{-1}
Complex mixtures:	
IPL Schneider's Drosophila medium Excell	Different cell culture media used as basic media.
Trifolium pratensis *Impatiens sp.* *Stachys vulgaris*	Plant extracts, employed in various concentrations.
Manduca conditioned media (Univ. of Washington/Arizona)	Different concentrations, also in combination with HMW or LMW fractions from hemolymph.
Redbull	
Vitamin pill (water soluble part in 50 ml)	2-100 µl
Vitamins, hormones, rest:	
Vitamin E	0.215, 2.15, 21.5 mg x ml^{-1}
b-Carotene	0.269, 2.69, 26.9 mg x ml^{-1}
Ascorbic acid (Vit. C)	0.008-0.8 mg x ml^{-1}
Vitamin A (acetate)	0.164, 1.64, 16.4 mg x ml^{-1}
Vitamn D3	0.192, 1.92, 19.2 mg x ml^{-1}
N-Acetyldopamin	0.1, 1, 3.76 nmol
EDTA (+ hemolymph)	0.5, 2.5, 5, 10 mM
Dibutyryl-cAMP	0.01, 0.1, 1 mM
Trehalose 6-Phosphate	10 µg/ml - 3mg x ml^{-1}
Juvenile hormone	1, 10, 100 ppm
Zeatin	0.01-10 µg x l^{-1}
Zeatin Riboside	0.01-10 µg x l^{-1}

CHAPTER 5

In vitro cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements and sporulation

Florian M. Freimoser[1]
Anne Grundschober[1]
Markus Aebi
Urs Tuor[2]

Eidgenössische Technische Hochschule ETH
Mikrobiologisches Institut
Schmelzbergstr. 7
CH - 8092 Zürich, Switzerland

Mycologia, 92(2), 2000, p. 208-215
Copyright © 2000, The Mycological Society of America,
Lawrence, KS 66044-8897

Accepted for publication November 3, 1999.
[1]Both authors contributed equally to this work.
[2]Corresponding author, email: tuor@micro.biol.ethz.ch

In vitro cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements and sporulation

Entomophthora thripidum Samson, Ramakers & Oswald (Zygomycota, Entomophthorales) was isolated from _Thrips tabaci_ Lindeman (Thysanoptera, Thripidae; onion or potato thrips). Vegetative fungal cells were released into culture medium by opening surface sterilized insects and multiplied in Grace's insect tissue culture medium supplemented with yeastolate, lactalbumin hydrolysate and 10% fetal bovine serum (FBS). These cells grew as protoplasts (no staining for cell wall) and aggregated to form pellets that were only observed _in vitro_. After exhaustion of the medium, walled mycelium formed which produced forcibly ejected spores when transferred onto water agar.

INTRODUCTION

The genus *Entomophthora* (Zygomycota, Entomophthorales) encomposes a group of entomopathogenic fungi characterized by their campanulate primary spores. These spores have an apical point, contain two up to 40 nuclei and are surrounded by a halo when ejected (Remaudière & Keller, 1980; Keller, 1987). This halo, or protoplasm, consists of mucilaginous material present in *Entomophthora* species between an inner and outer wall layer of the spore (Eilenberg *et al.*, 1986). Primary spores produce secondary spores that do not have a clearly visible protoplasm (Keller, 1987). It has been suggested that the secondary spore might be the main infective structure in the genus *Entomophthora* (Bellini *et al.*, 1992; Eilenberg *et al.*, 1995). The type species is *Entomophthora muscae* (Cohn) Fresenius which was described as early as 1855 as a pathogen of house flies (*Musca domestica* L.) (Cohn, 1855). Other frequent species are *E. planchoniana* Cornu in Europe (Wilding, 1975; Remaudière *et al.*, 1981) and *E. chromaphidis* Burger & Swain in the USA and Australia (Humber & Feng, 1991), both naturally occurring pathogens of aphids (Homoptera, Aphididae). *Entomophthora thripidum* Samson, Ramakers & Oswald grows in the abdomen of *Thrips tabaci* Lindeman (Thysanoptera, Thripidae; onion or potato thrips) until it breaks through the cuticle and

discharges its primary spores while the insect is still alive. It has only been described once in the Netherlands (Samson *et al.*, 1979).

Fungi in general and Entomophthorales in particular are promising as biocontrol agents because of their active invasion and attack of insects and their potential to cause epizootics (Leathers *et al.*, 1993). A special feature of Entomophthorales are the forcibly discharged spores which facilitate the spread of the disease. The demanding nutritional requirements for *in vitro* cultivation are a major drawback to the commercial development of this highly specialized group of insect pathogens (Samson *et al.*, 1988).

Thrips tabaci is an important cosmopolitan and polyphagous pest that causes feeding damage and transmits plant viruses (Lewis, 1997). Thrips in general are difficult to manage and no specific insecticides exist for their control (Lewis, 1997). This is one reason why these pest insects are excellent targets for biological control.

In this publication we describe for the first time the nutritional requirements for the *in vitro* cultivation of the thrips pathogen *E. thripidum,* the cell morphology *in vitro* as well as the sporulation characteristics of this fungus on artificial media.

MATERIALS AND METHODS

Collection:-- Infected *T. tabaci* were collected in 1998 by Axel Weber (Institute for Plant Protection in Horticulture, Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany) from a laboratory colony. An infection rate approaching 95% was observed in the thrips colonies under conditions of high humidity (A. Weber pers comm). Fungal identification to species was done by Dr. H. Sierotzki in our laboratory using the original description of Samson et al (1979). The isolate we used in this study was deposited in the ARSEF culture collection (USDA-ARS, Ithaca, NY) as ARSEF 5868.

Isolation in liquid medium:-- We followed the same isolation protocol previously described for the isolation of *Neozygites parvispora* (Grundschober *et al.*, 1998). Briefly, infected but still living thrips (adults and larvae) were surface sterilized by dipping in ethanol (70% v/v) for 1 min, NaOCl (3% w/w) for 3 min and twice in sterile water (at least 3 min each). The surface sterilized insects were crushed in 50 μL cultivation medium (in 24-well culture plates) to release the fungal cells. These preparations were incubated at 20 C, in the dark and checked regularly on an inverse microscope for evidence of cell multiplication.

When cell division was observed, fresh medium was added to the multiwell plates, gradually increasing the culture volume in two to four steps to reach a maximum volume of 500 µL. At this point cultures were transferred to a shaking incubator (20 C, in the dark, 160 rpm) and subcultured once per week.

Cultivation in liquid medium:-- *Entomophthora thripidum* cells were routinely grown in 24-well culture plates containing 1 mL medium per well. Plates were held at 20 C in the dark and agitated at 160 rpm. Cultures were homogenized by gentle pipetting and 25 to 50 µL of the old culture were subcultured into 1 mL of fresh medium every week.

The standard cultivation medium was based on Grace's insect tissue culture medium 2X supplemented with lactalbumine hydrolysate and yeastolate (Gibco No. 11667). It was diluted to 1X by adding 10% heat inactivated fetal bovine serum (FBS; Gibco) and 40% water. This medium had a pH of 6.5 and an osmolarity of 380 mOsm.

For comparison of different media and the determination of a growth curve, the fungus was grown in 48-well culture plates containing 500 µL medium per well. Each well was inoculated with 10^4 cells/mL derived from 5- to 7-d-old cultures, previously washed once with 350 mM sucrose. The plates were kept at 20 C in the dark and shaken at 160 rpm. Growth was determined in the presence and absence of FBS (pH 6.5 and 5.8 respectively, osmolarity 380 mOsm). Cell densities were measured daily in independent samples (4 replicates per sampling date) over a period of 15 days except for days 1, 11, 13, 14, when no samples were taken. The experiment was performed twice.

In the growth experiments we compared Grace's medium with and without supplements (Gibco No. 11667 and 21590) in combination with FBS and hemolymph. Hemolymph was obtained from 5th instar *Manduca sexta* (L.) as described in Grundschober et al (1998). The liquid GLEN medium containing 0.4% glucose, 0.5% yeast extract, 0.65% lactalbumin hydrolysate and 0.77% NaCl (Beauvais & Latgé, 1988) with 10% FBS was also tested. These tests were evaluated after 7d.

Growth on solid medium, spore formation and characterization:-- Growth trials on solid medium were performed on Sabouraud-dextrose agar with egg yolk (SDAEY: 3% Sabouraud dextrose, 1.7% agar and 8% egg yolk; (Keller, 1987). Plates were held in a dark box containing wet tissue paper at 20 C. To induce sporulation, fungal material was plated on water agar or SDAEY and incubated in humid boxes at 20 C. To determine spore size, spores were showered onto glass slides and measured using light microscopy.

Determination of cell and spore sizes:-- Cells and *in vitro* produced spores were observed under a Zeiss Axiophot light microscope at x400. Pictures were projected onto a screen via a digital camera (Hamamatsu color chilled 3CCD camera and camera controller C5810). Cells were measured on the screen using a calibrated scale. Cells were considered as long, when they were at least twice as long as wide.

Determination of cell densities:-- The cell density of the inoculum used in the various tests was determined with conventional hemocytometer counts. Growth of cells in the liquid media was quantified using a colorimetric assay based on MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) as described by Freimoser et al (1999).

Staining:-- Nuclei were stained with DAPI (4,6-Diamidino-2-phenylindole). Cells were first suspended in 10 µL DAPI solution (0.05 mg/mL). Ethanol (2 µL, 1:5 v/v) was added, and cells were observed after incubation of at least 10 minutes at room temperature. Cell walls were stained using Blancophor (Bayer Cat. No. 705 0129 127 01). Blancophor solution (1 mg/mL) was added to cells in culture medium (2 µL Blancophor per 20 µL cell suspension). All preparations were observed by fluorescence microscopy on a Zeiss Axiophot with a 365 nm excitation filter, 395 nm beam splitter and 420 nm barrier filter.

Storage of cells:-- Cells were concentrated by centrifugation (30g, 15 min, 20 C) and resuspended in cryotubes (Nunc) with fresh FBS containing 10% DMSO (Sly & Grubb, 1979). The cryotubes placed in a polystyrene box containing paper pading were held at -80 C overnight to allow slow cooling and then stored above liquid nitrogen or at -80 C (Zwerner *et al.*, 1979).

Statistical analysis:-- For comparison of growth and sporulation characteristics in different media, data were subjected to an analysis of variance (ANOVA) using Tukey's Honest Significant Difference (HSD) procedure (SAS Institute, 1990). Statistical differences in the figure and text were expressed at the 5% significance level.

RESULTS

Isolation:-- Fungal cells from surface sterilized thrips started to grow shortly after release into supplemented Grace's insect cell culture medium with FBS and hemolymph. Addition of hemolymph in the medium was not necessary for continued cultivation of this fungus and later experience showed that hemolymph is not necessary for the isolation of this fungus either (results not shown).

Morphology and growth in liquid culture:-- In liquid culture *E. thripidum* formed long club-shaped cells as well as shorter rod shaped and round cells that were produced by budding (Fig. 1A). Spindle-shaped cells were also observed. They remained connected with the mother cell by a thin thread, thus forming chains of spindle-shaped cells (Fig. 1B). All of these cell forms did not stain with Blancophor and burst when suspended in distilled water, suggesting that they were protoplasts lacking a cell wall.

Protoplasts remained connected and aggregated in spherical pellets (Fig. 1B, C, D). These pellets increased in diameter by growth at the periphery, where

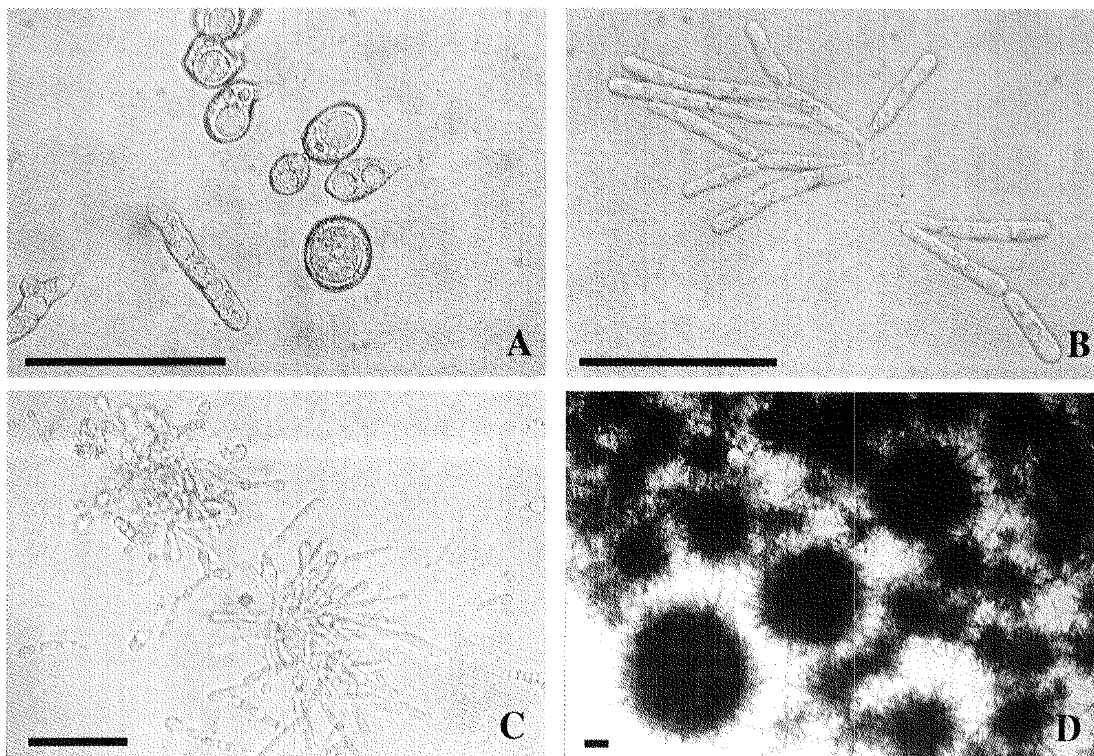


Fig. 1. Protoplast morphology and stages in pellet formation of *E. thripidum*. A. Single long and round shaped protoplasts of *E. thripidum*. B. Protoplasts connected with each other by a thin thread. C. Early stage in protoplasts aggregation. D. Late stage of aggregate showing dense pellets. Bars: A= 50 µm; B, C, D= 100 µm.

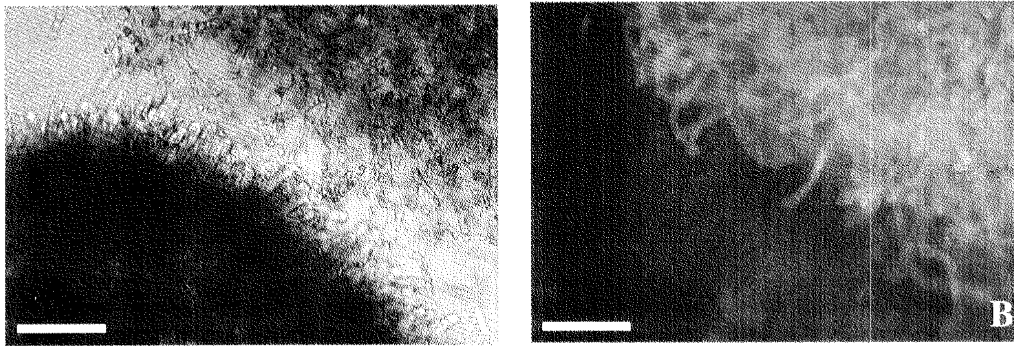


Fig. 2. Distinction of protoplasts and walled cells from liquid cultures by cell wall staining. Protoplast pellets (lower left corner; see fig. 1D) and mycelial pellets (upper right corner) from separate cultures of *E. thripidum* of different age were mixed and stained with Blancophor. A. Preparation under the light microscope shows protoplasts and mycelium. B. Fluorescent light reveals that only mycelium stains with Blancophor. Protoplasts do not stain. Bars: 100 µm.

the rod- and club-shaped cells were located. The center of the pellets consisted of round cells. When *E. thripidum* was maintained without replenishing the growth medium for at least 2 wk it eventually formed fluffy mycelium. This was accompanied by a change in cell morphology. Instead of short club-shaped cells, long and septated hyphae developed. They contained numerous empty compartments and stained strongly with Blancophor indicating the presence of a cell wall (Fig. 2).

Entomophthora thripidum protoplasts were isolated in the same medium as *N. parvispora*, i. e. supplemented Grace's insect cell culture medium, FBS and hemolymph (Grundschober *et al.*, 1998), in which they readily started to grow. We tested the effect of these amendments on cell density (Fig. 3). The addition of 10 or 20% FBS, 10% hemolymph or 10% of both FBS and hemolymph resulted in higher cell densities. A reduction of the FBS content to a concentration of 10% did not significantly reduce growth, whereas growth in a medium containing 5% FBS was significantly lower and growth levels were indistinguishable from that in a medium containing no additives. Inclusion of hemolymph in the medium had a similar influence on cell growth as FBS and it was possible to substitute one with the other without affecting cell growth or density. The supplementation of the media with lactalbumin-hydrolysate and yeastolate on the other hand significantly increased growth of *E. thripidum*. For both supplemented and non-supplemented media, addition of either FBS or hemolymph stimulated cell growth, and a slight decrease in cell density was observed when they were added in combination. Additionally the fungus also grew in GLEN medium supplemented with 10% FBS (data not shown). In all media, irrespective of the presence of FBS, cells did not stain with Blancophor suggesting that they did not have a cell wall (except the mycelia described above).

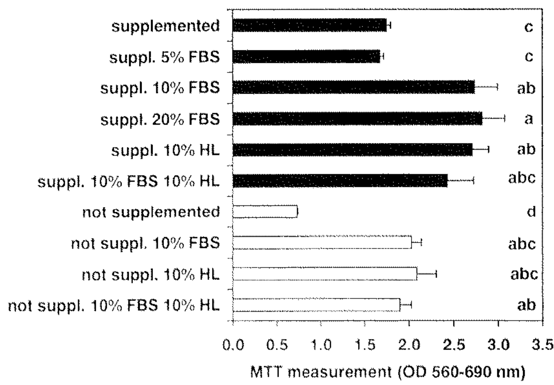


Fig. 3. Comparison of growth in different media after cultivation for seven days. Solid bars represent media based on Grace's medium supplemented with lactalbumin hydrolysate and yeastolate, and containing 5, 10 or 20% FBS and/or 10% hemolymph (HL). White bars represent growth in media based on unsupplemented Grace's medium to which 10% FBS and/or 10% hemolymph has been added. Different letters indicate statistically significant differences between media (ANOVA with Tukey's HSD test, $P < 0.05$).

The maximal cell density reached under our culture conditions in supplemented Grace's insect cell culture medium with 10% FBS was $1.2*10^6$ cells per mL. After a short lag-phase the fungus grew exponentially. Thereafter growth slowed down, and the maximum cell density was reached after 7 d (Fig. 4). The maximum specific growth rate was 1.3 d^{-1} equaling a doubling time of 0.5 d. After about 7 d in an optimal medium, cells started to degenerate and the cell density decreased (Fig. 4). This was accompanied by an increasing proportion of round cells. During the exponential phase a higher proportion of the cells had an elongated shape (Table I). In the same medium lacking FBS growth was slower, not exponential, and resulted in comparably low cell densities (Fig. 4). Omitting FBS also led to a delayed increase in the ratio of long to round cells and to an earlier decline in this ratio (data not shown).

Growth on solid medium and sporulation:-- Mycelium or protoplast pellets that were put on SDAEY plates grew extremely slowly and did not cover the plate within 5 wk. On solid media *E. thripidum* grew vertically rather than horizontally, forming vaults and crevasses (Fig. 5). Radial growth was marginal and slow.

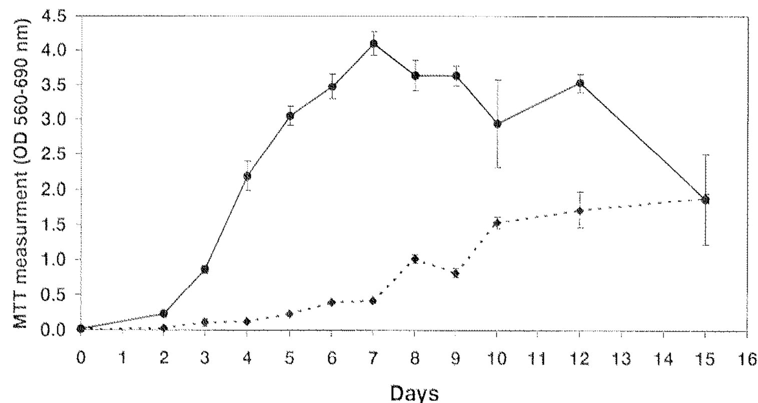


Fig. 4. Growth curve of *E. thripidum* protoplasts in two different media: supplemented Grace's with FBS (-●-), supplemented Grace's without FBS (- -◆- -). Error bars = se.

Table I. Characterization of cell morphology and density at different growth phases after 0, 4 and 12 days of culture in Grace's insect cell culture medium supplemented with lactalbumin hydrolysate, yeastolate and 10% FBS (mean ± SE).

	Lag phase (0 d)	Log phase (4 d)	Stationary phase (12 d)
Cell density (cells/mL)	$(1.7\pm0.5)^{*}\ 10^{4}$	$(3.8\pm0.2)^{*}\ 10^{5}$	$(9.7\pm0.5)^{*}\ 10^{5}$
Length of long cells (µm)	31±2	49±3	62±4
Length of round cells (µm)	22±2	25±2	25±2
Proportion of long cells	0.4±0.1	0.65±0.06	0.34±0.04
Proportion of round cells	0.6±0.2	0.35±0.03	0.66±0.04

Cells produced *in vitro* were able to sporulate after transfer onto water agar. Protoplasts (not staining with Blancophor) that were put on water agar produced only very few spores. If mycelia were plated on water agar, primary spores were produced overnight. However, spores were only formed on dry areas of the mycelial biomass; on surface areas coated with a film of water, sporulation was not observed. Primary spores were forcibly ejected and surrounded by protoplasm (Fig. 6A). They contained 3 to 6 nuclei, had an average length of 15.5 ± 0.2 µm and a width of 10.9 ± 0.1 µm (105 spores from 3 different isolates of *E. thripidum* counted). The shape of the primary spores was campanulate, typical for the genus *Entomophthora* (Keller, 1987). The primary spores formed secondary spores that were also ejected but which were not surrounded by protoplasm (Fig. 6B). The secondary spores also contained 3 to 6 nuclei and were slightly smaller. Their average size was 10.6 ± 0.2 µm long, 9.5 ± 0.1 µm wide (58 spores from 3 different isolates of *E. thripidum* counted). Protoplasts, in comparison, contained up to 16 nuclei. *Entomophthora thripidum* lost its capacity to sporulate after prolonged subculturing. After 2 mo of weekly subcultures, sporulation was unpredictable, occurring irregularly after plating on egg yolk medium (SDAEY) and after 1-4 wk of incubation.

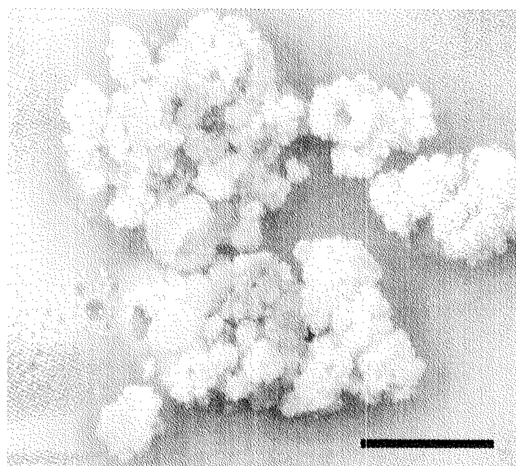


Fig. 5. Formation of a fungal colony upon transfer of mycelium from liquid cultures onto a SDAEY agar plate (6 wk old). Growth was very slow. Bar: 1 cm.

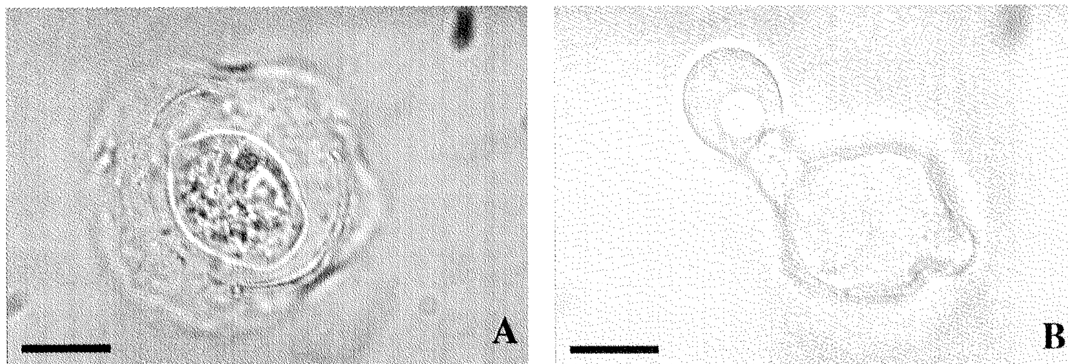


Fig. 6. Primary and secondary spores of *E. thripidum* produced in vitro. A. Primary spore surrounded by a protoplasm. B. Formation of a secondary spore from a primary spore.
Bar: 10 µm.

Maintenance:-- After storage (3 mo) in the vapor phase above liquid nitrogen (approx -160 C), or at –80 C new cultures of *E. thripidum* could be started from the frozen material.

DISCUSSION

We report the isolation of *E. thripidum*, from a collapsed thrips culture in Germany. Samson et al (1979) found this fungal species regularly on *T. tabaci* over a 3 yr period in glasshouses in the Netherlands. We are aware of only one additional observation from glasshouses in Denmark (J. Eilenberg pers comm). The lack of literature on *E. thripidum* suggests that it may be a rare pathogen of thrips, but this could also be a result of its unobtrusiveness and lack of a consistent, targeted monitoring program.

In vitro cultures of *E. thripidum* were started with vegetative cells from infected but still living insects, an approach that yielded cultures of another challenging pathogen, *N. parvispora* (Grundschober *et al.*, 1998). The authors who first described *E. thripidum* stressed that it was not possible to isolate this species from sporulating cadavers (Samson *et al.*, 1979). Our findings seem to confirm other observations that many obligatory parasitic fungi can only be isolated when starting from vegetative material and not from spores (Humber, 1994).

The nutritional requirements of *E. thripidum* were not as demanding as those of *N. parvispora* since hemolymph was not necessary for growth. Nevertheless, the addition of FBS was necessary to promote fast and dense development. The function of FBS could be replaced by hemolymph. This is similar to findings for insect cell cultures where hemolymph, once used as a supplement in culture media, was replaced by 10% FBS (Ferkovich & Oberlander, 1991; Schlaeger, 1996). One function of FBS in cell cultures, mainly through serum albumin, is

that of a carrier for fatty acids (Nilausen, 1978; Spector, 1986). FBS may also stabilize the membranes of the protoplasts as suggested for *Entomophaga aulicae* (Beauvais & Latgé, 1988). Supplementation with lactalbumin hydrolysate and yeastolate increased growth as in insect cell culture (Schlaeger, 1996), but did not induce a significantly higher growth response to warrant a replacement of FBS.

We showed that in liquid culture, *E. thripidum* formed protoplasts which occur in the life cycle of several entomopathogenic fungi (Butt *et al.*, 1981; Nolan, 1985), enabling these pathogens to evade immune recognition by the host (Beauvais *et al.*, 1989). The protoplasts of *E. aulicae* (=*Entomophthora egressa*) were observed to develop in defined stages (Nolan, 1985). But similar growth development was not seen in *Entomophthora muscae* (Latgé *et al.*, 1988) or *E. thripidum,* and the only different stages observed were the round- and long-shaped protoplasts. Protoplasts in infected thrips mostly had an elongated shape and during the exponential growth phase in culture, a higher proportion of cells displayed this long shape. We think that the long and round protoplasts do not reflect two equal growth morphologies, but rather that the cells round up when growth conditions are not optimal anymore. Under growth conditions where nutrients are limited, hyphal bodies of *N. parvispora* also became rounded (Grundschober *et al.*, 1998).

The aggregates formed by the protoplasts of *E. thripidum* seem to be an artifact of *in vitro* cultivation as they were never observed *in vivo*. These pellets were often bigger than whole thrips. Cell lysis in the center of the aggregates, which can be caused by nutrient and oxygen limitations (Prosser & Tough, 1991), is a drawback of aggregates. The consequence is a lower final cell density, a disadvantage with respect to the possible production of *E. thripidum* for use as a biological insecticide. On the other hand, the pellet form could protect the fragile protoplasts against shear forces. Pellet growth is often preferred for process scale-up, because it facilitates processing and handling (Prosser & Tough, 1991). Another consequence of growth as pellets is the alteration of growth kinetics in comparison to the exponential growth of individual cells. It is assumed that pellets grow exponentially until their radii reach a size where diffusion of oxygen or nutrients is limited to the periphery; growth will then follow cube-root kinetics (Pirt, 1966). Our results also indicate that after an early exponential growth period growth of *E. thripidum* followed cube-root kinetics, but more data and experiments are required to prove this.

After aging protoplast cultures of *E. thripidum* and without adding fresh medium, the cells started to form a cell wall, and mycelia developed. The same observation is reported for *E. muscae* (Latgé *et al.*, 1988). In contrast,

E. aulicae forms walled hyphal bodies in a medium lacking FBS, and protoplasts are only formed if FBS is added (Beauvais & Latgé, 1988). This dependency upon FBS could not be observed for *E. muscae* or for *E. thripidum,* suggesting that the genus *Entomophthora* responds to other mechanisms for the control of cell wall synthesis. Mycelia that formed after starvation could be transferred onto water agar where they produced spores after overnight incubation, that were identical to those produced *in vivo*. They had the same size and number of nuclei as spores described by Samson et al (1979) in the original report. As has already been observed for *N. parvispora,* sporulation of *E. thripidum* occurred only in an aerial environment. Liquid cultures never yielded spores, and the presence of a water film on the mycelium, when plated on agar, also suppressed sporulation. Since mycelia produced by *E. thripidum* could survive and sporulate when removed from the cultivation medium, it was not necessary to entrap it in an alginate matrix as for hyphal bodies of *N. parvispora* (Grundschober *et al.*, 1998).

After repeated subculture, cells lost their capacity to sporulate overnight. Such phenotypical alteration during serial subculturing is a common phenomenon in fungi. Virulence, sporulation or pathogenicity are often affected (Hajek *et al.*, 1990; Humber, 1997). These losses may be recovered by passaging through an insect host (Hayden *et al.*, 1992; Goettel & Inglis, 1997). In our case isolates were stored at ultra-low temperatures in the hope of conserving their original properties.

Future work will define the nutritional and physiological requirements for sporulation of *E. thripidum* and it will be necessary to develop a bioassay with *T. tabaci*. In conclusion our work with two pathogens of *T. tabaci* has shown that Entomophthorales that were thought to be obligate biotrophs can now be cultured in complex liquid media. However, to promote further development of these pathogens as biopesticides, means of inducing sporulation in artificial culture remains a primary objective.

ACKNOWLEDGMENTS

We thank A. Weber for kindly collecting and sending infected thrips, enabling this study in the first place, Dr. H. Sierotzki for determination of the fungus, M. Eggli FAW Wädenswil (Zürich) for providing *T. tabaci* and Dr. P. Shah for help in statistical analysis and reading of the manuscript. This work is supported by the Kommission für Technologie und Innovation (KTI grant 3202.1), Novartis AG, Basel, and the ETH Zürich.

REFERENCES

Beauvais, A. & Latgé, J. P. (1988). A simple medium for growing entomophthoralean protoplasts. *J Invertebr Pathol* **51**, 175-178.

Beauvais, A., Latgé, J. P., Vey, A. & Prevost, M. C. (1989). The role of surface components of the entomopathogenic fungus *Entomophaga aulicae* in the cellular immune response of *Galleria mellonella* (Lepidoptera). *J Gen Microbiol* **135**, 489-498.

Bellini, R., Mullens, B. A. & Jespersen, J. B. (1992). Infectivity of two members of the *Entomophthora muscae* complex (Zygomycetes: Entomophthorales) for *Musca domestica* (Dipt.: Muscidae). *Entomophaga* **37**, 11-19.

Butt, T. M., Beckett, A. & Wilding, N. (1981). Protoplasts in the *in vivo* life cycle of *Erynia neoaphidis*. *J Gen Microbiol* **127**, 417-421.

Cohn, F. (1855). *Empusa muscae* und die Krankheit der Stubenfliegen. *Hedwigia* **10**, 57-61.

Eilenberg, J., Bresciani, J. & Latgé, J. P. (1986). Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora*. *J Invertebr Pathol* **48**, 318-324.

Eilenberg, J., Bresciani, J., Olesen, U. & Olson, L. (1995). Ultrastructural studies of secondary spore formation and discharge in the genus *Entomophthora*. *J Invertebr Pathol* **65**, 179-185.

Ferkovich, S. M. & Oberlander, H. (1991). Growth factors in invertebrate *in vitro* culture. *In Vitro Cell Dev Biol* **27A**, 483-486.

Freimoser, F. M., Jacob, C., Tuor, U. & Aebi, M. (1999). The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay is a fast and reliable method for the colorimetric determination of fungal cell densities. *Appl Environ Microbiol* **65**, 3727-3729.

Goettel, M. S. & Inglis, G. D. (1997). Fungi: Hyphomycetes. In *Manual of techniques in insect pathology*, pp. 213-249. Edited by Lacey, L. A. San Diego: Academic Press.

Grundschober, A., Tuor, U. & Aebi, M. (1998). *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *Syst Appl Microbiol* **21**, 461-469.

Hajek, A. E., Humber, R. A. & Griggs, M. H. (1990). Decline in virulence of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) with repeated *in vitro* subculture. *J Invertebr Pathol* **56**, 91-97.

Hayden, T. P., Bidochka, M. J. & Khachatourians, G. G. (1992). Entomopathogenicity of several fungi toward the English grain aphid (Homoptera: Aphididae) and enhancement of virulence with host passage of *Paecilomyces farinosus*. *J Econ Entomol* **85**, 58-64.

Humber, R. A. (1997). Fungi: Preservation of cultures. In *Manual of Techniques in Insect Pathology*, pp. 269-279. Edited by Lacey, L. A. San Diego: Academic Press.

Humber, R. A. (1994). Special considerations for operating a culture collection of fastidious fungal pathogens. *J Indust Microbiol* **13**, 195-196.

Humber, R. A. & Feng, M. G. (1991). *Entomophthora chromaphidis* (Entomophthorales): The correct identification of an aphid pathogen in the Pacific Northwest and elsewhere. *Mycotaxon* **41**, 497-504.

Keller, S. (1987). Arthropod-pathogenic Entomophthorales of Switzerland. I *Conidiobolus*, *Entomophaga* and *Entomophthora*. *Sydowia* **40**, 122-167.

Latgé, J. P., Eilenberg, J., Beauvais, A. & Prevost, M. C. (1988). Morphology of *Entomophthora muscae* protoplasts grown *in vitro*. *Protoplasma* **146**, 166-173.

Leathers, T. D., Gupta, S. C. & Alexander, N. J. (1993). Mycopesticides: Status, challenges and potential. *J Indust Microbiol* **12**, 69-75.

Lewis, T. (1997). Chemical control. In *Thrips as Crop Pests*, pp. 567-593. Edited by Lewis, T. Wallingford: CAB International.

Lewis, T. (1997). Pest thrips in perspective. In *Thrips as Crop Pests*, pp. 1-13. Edited by Lewis, T. Wallingford: CAB International.

Nilausen, K. (1978). Role of fatty acids in growth-promoting effect of serum albumin on hamster cells in vitro. *J Cell Physiol* **96**, 1-13.

Nolan, R. A. (1985). Protoplasts from Entomophthorales. In *Fungal Protoplasts*, pp. 87-112. Edited by Peberdy, J. F. & Ferenczy, L. New York: Marcel Dekker Inc.

Pirt, S. J. (1966). A theory of the mode of growth of fungi in the form of pellets in submerged culture. *Proc Roy Soc London, B Biol* **166**, 369-73.

Prosser, J. I. & Tough, A. J. (1991). Growth mechanisms and growth kinetics of filamentous microorganisms. *Crit Rev Biotechnol* **10**, 253-274.

Remaudière, G. & Keller, S. (1980). Systematic revision of the genera of Entomophthoraceae with entomopathogenic potential. *Mycotaxon* **11**, 323-338.

Remaudière, G., Latgé, J. P. & Michel, M. F. (1981). Comparative ecology of Entomophthoraceae pathogenic to aphids in coastal and inland France. *Entomophaga* **26**, 157-178.

Samson, R. A., Evans, H. C. & Latgé, J. P. (1988). *Atlas of Entomopathogenic Fungi*: Springer Verlag.

Samson, R. A., Ramakers, P. M. J. & Oswald, T. (1979). *Entomophthora thripidum*, a new fungal pathogen of *Thrips tabaci*. *Can J Bot* **57**, 1317-1323.

SAS Institute, I. (1990). *SAS/STAT user's guide*, Version 6, 4th ed. edn. Cary, NC: SAS Institute, Inc.,.

Schlaeger, E.-J. (1996). Medium design for insect cell culture. *Cytotechnology* **20**, 57-70.

Sly, W. S. & Grubb, J. (1979). Isolation of fibroblasts from patients. In *Methods in Enzymology*, pp. 444-450. Edited by Jakoby, W. B. & Pastan, I. H. London: Academic Press.

Spector, A. A. (1986). Structure and lipid binding properties of serum albumin. In *Plasma Proteins; Preparation, Structure and Molecular Biology (Part A)*, pp. 320-339. Edited by Segrest, J. P. & Albers, J. J. Orlando, FL: Academic Press.

Wilding, N. (1975). *Entomophthora* species infecting pea aphids. *Trans Roy Entomol Soc* **127**, 171-183.

Zwerner, R. K., Wise, K. S. & Action, R. T. (1979). Harvesting the products of cell growth. In *Methods in enzymology*, pp. 221-229. Edited by Jakoby, W. B. & Pastan, I. H. London: Academic Press.

CHAPTER 6

Regulation of hyphal growth and sporulation of the insect pathogenic fungus *Entomophthora thripidum* *in vitro*

Florian M. Freimoser
Anne Grundschober
Urs Tuor
Markus Aebi[1]

Institute of Microbiology
ETH Zürich, Schmelzbergstr. 7
CH-8092 Zürich, Switzerland

Submitted for publication

[1]Author for correspondence: M. Aebi. Tel.: ++41 +1 632 64 13. Fax: ++41 +1 632 11 48.
e-mail: aebi@micro.biol.ethz.ch

Regulation of hyphal growth and sporulation of the insect pathogenic fungus *Entomophthora thripidum in vitro*

Entomophthora thripidum is an obligate biotrophic insect pathogenic fungus that occurs in two forms within the hemocoel of _Thrips tabaci_: protoplasts are observed in the first phase of vegetative growth, whereas hyphal growth is observed prior to penetration through the insect cuticle and spore formation at the insect surface. _In vitro,_ the differentiation to hyphal growth was detected in liquid culture 10 to 20 d after inoculation. Hyphal growth was a prerequisite for the subsequent formation of infectious spores. _E. thripidum_ secreted a factor that autoinduced the differentiation to hyphal growth in cultures that did not differentiate to this form otherwise. The production of this "mycelium factor" was induced upon nitrogen limitation in the growth medium. Its molecular weight was bigger than 30 kDa and it was sensitive to digestion with trypsin. The discovery of the activity inducing hyphal growth made possible the reliable production of spores, the infection of _T. tabaci_ and the consecutive reisolation of the fungus from the infected insects.

INTRODUCTION

The life cycle of obligate biotrophic insect pathogenic fungi can be separated into three distinct steps; the infection of the host, vegetative growth within the host insect and the breaking through the insect cuticle in order to sporulate and to reinfect. The infection process, starting with the attachment of a spore to the cuticle of a host insect, the germination of this spore and the consecutive penetration through the cuticle, has been studied thoroughly and many steps have been characterised at the molecular level (reviewed in Charnley, 1989; St. Leger, 1993). Once the fungus has overcome the barrier of the insect's cuticle it multiplies within the body of the host (reviewed in Samson *et al.*, 1988; Hajek & St. Leger, 1994). This invasion of the insect body is considered the last step in fungal pathogenesis and usually results in the death of the infected host (Samson *et al.*, 1988). The following step in the life cycle, the penetration

through the insect cuticle by outward growth and subsequent spore formation, has received less attention.

The induction of the sporulating stage brings about fundamental changes in the morphology of the fungus. Insect pathogenic fungi belonging to the order of the Entomophthorales often grow in the form of discrete cells with some species multiplying as protoplasts (Samson *et al.*, 1988). For sporulation fungal cells grow coordinatedly outward, break through the insect's cuticle and spores are formed at the tip of hyphae that are surrounded by a cell wall (Brobyn & Wilding, 1977). Sporulation is timed to assure that all cells form spores simultaneously before the insect cadaver is overgrown by saprophytic and opportunistic bacteria or fungi. Regulated sporulation has to occur in those species of Entomophthorales that sporulate from insects that are still alive or that only break through the cuticle at defined places (summarised in Humber, 1984; Evans, 1989).

Entomophthora thripidum (Zygomycetes: Entomophthorales) is a pathogen of *Thrips tabaci* (Thysanoptera: Thripidae; onion or potato thrips) (Samson *et al.*, 1979), a common pest insect in vegetables and ornamentals (Lewis, 1997). This fungus is especially noteworthy for its localised sporulation from the abdomen of still living thrips (Samson *et al.*, 1979). *In vitro E. thripidum* grows and multiplies in the form of irregularly shaped protoplasts that aggregate to form pellets (Freimoser *et al.*, 2000). After prolonged cultivation in the same growth medium the protoplasts of *E. thripidum* will differentiate to hyphae that are surrounded by a cell wall. The pellets consisting of these hyphae are referred to as mycelium (Freimoser *et al.*, 2000). Upon transfer onto water agar the mycelium formed by these hyphae will produce infectious spores. *In vivo*, within the thrips, the protoplasts of *E. thripidum* also undergo the switch from protoplast to hyphal growth and sporulation can occur while the insect is still alive (Samson *et al.*, 1979; Freimoser *et al.*, 2000).

In vitro the switch from protoplast to hyphal growth was a prerequisite for sporulation. It was further observed that *E. thripidum* autoinduced hyphal growth and mycelium formation by a secreted factor. This finding enabled the reliable production of infectious spores and the completion of the life cycle of *E. thripidum* under laboratory conditions.

MATERIAL AND METHODS

Isolates and cultivation:-- For all experiments *E. thripidum* (isolates ARSEF 5868, 6517, 6518, 6519, 6520, 6525 and 6521 and 6522, two reisolates of ARSEF 6518; all deposited in the ARSEF Collection of Entomopathogenic

Fungal Cultures, USDA-ARS Plant Protection Research Unit, US Plant, Soil, and Nutrition Laboratory, Tower Road, Ithaca, New York, 14853-2901, USA) was grown in 48-well or 24-well microtiter plates in 500 µl or 1 ml of culture medium, respectively. The standard growth medium was Grace's insect cell culture medium supplemented with lactalbumine hydrolysate and yeastolate (GIBCO) and 10% foetal bovine serum (FBS, GIBCO). Alternatively, supplemented GLEN medium, containing 0.4% glucose, 0.5% yeast extract, 0.65% lactalbumin hydrolysate, 0.77% NaCl (all w/v) and 10% FBS (v/v) (Beauvais & Latgé, 1988) was used. This medium made it possible to omit the carbon (glucose) and/or the nitrogen sources (yeast extract and lactalbumin hydrolysate) in order to determine their influence on the formation of mycelium. The cultures were inoculated with approx. 10^4 cells x ml^{-1}, kept in the dark at 20°C and shaken at 160 rpm. Reisolation was performed as described by Grundschober et al. (1998) from *Thrips tabaci* that had been infected by the method developed for *Neozygites parvispora* (Grundschober, 2000).

Morphological observations and sporulation:-- Different isolates of *E. thripidum* were examined daily under an inverse microscope for 20 d to follow the process of mycelium formation. The presence or absence of a cell wall was determined by addition of 1 µl of calcofluor (0.1 mg/ml final conc.) to 10 µl of fungal culture followed by detection with fluorescence microscopy using a Zeiss Axiophot with a 365 nm excitation filter, 395 beam splitter and 420 nm barrier filter. All cultures were grown in duplicate.

To induce spore formation fungal cultures were transferred onto 1.5% water agar and incubated in a humid, translucent box overnight at 20°C in light. The ability to form spores was assessed qualitatively.

Analytical measurements:-- The density of living cells was determined by the MTT assay as described elsewhere (Freimoser *et al.*, 1999). The glucose concentration in the supernatant growth medium was measured enzymatically (SIGMA DIAGNOSTIC KIT, No. 16-20), while the concentration of total α-amino acids was determined colorimetrically by the OPA-method using leucine as a standard (Frister *et al.*, 1986). The pH was determined by using a pH electrode (METTLER TOLEDO, InLab® 423, Electrolyte 9811) and a pH meter (KNICK pH-meter 761, Calimatic) prior to freezing supernatants for further analysis.

All measurements were performed in 4 independent replicates and experiments were performed twice with different fungal isolates.

Mycelium formation in different media:-- To test the influence of medium compositions on the formation of mycelium the standard cultivation medium was removed after 7 d and replaced by the medium to be tested. Protoplast pellets of *E. thripidum* were aspired with a cut pipette tip and transferred to an eppendorf tube, where they sedimented. The supernatant was then removed, the new medium added and the cultures were again placed in a 48-well microtiter plate.

To determine the influence of carbon and nitrogen sources on mycelium formation GLEN medium where glucose and/or lactalbumin hydrolysate and yeastolate had been omitted was tested. To assess the activity of conditioned medium, it was filter sterilised and used to replace half of the culture medium of 7 d old *E. thripidum* cultures. Medium from cultures that had formed mycelium (CMM) was tested at different concentrations (100%, 50%, 25%, 12.5%) by removing only part of the old culture medium and replacing it with CMM. In one experiment the old medium was not changed, instead different volumes were removed in order to decrease the culture volume (and thus increasing the cell density) after 7 d by 20%, 40%, 60% or 80%.

In addition, the medium of a 7 d old test culture was replaced by a mixture of equal amounts of CMM and GLEN medium (either complete or without the C- and/or N-source). As a control only half of the medium of a 7 d old culture of *E. thripidum* was replaced with either CMM or one of the variations of the GLEN medium.

For all experiments 2 or 4 replicates were used and each experiment was performed twice with different isolates. In all cases a control was used where the culture medium was not changed.

Characterisation of factors from conditioned medium:-- To characterise the factor(s) that induced the switch to hyphal growth and mycelium formation conditioned medium was subjected to different treatments. CMM was either heated to 100°C for 10 min, extracted with equal volumes of chloroform, fractionated by centrifugation through membranes with 30 kDa, 10 kDa and 3 kDa cut off (CENTRICON concentrators, Amicon), concentrated 20-fold with the 30 kDa cut off filter or digested with 8000 U of trypsin (SIGMA; 37°C, 1.5 h). Trypsin was inactivated by the addition of trypsin inhibitor (4 mg, ~20 kDa, FLUKA).

RESULTS

Hyphal growth was a prerequisite for vegetative spore formation in vitro:-- *In vitro, E. thripidum* grew and multiplied as protoplasts. Hyphal growth and mycelium formation, concomitant with cell wall formation, was observed after prolonged cultivation without subculturing and usually occurred between 10 to 20 d after inoculation (Freimoser *et al.*, 2000). However, not all isolates were able to perform this differentiation process. The switch from protoplasts to hyphal growth was an irreversible process and affected all cells in the respective well (Fig. 1a-d).

Following transfer onto water agar mycelium of *E. thripidum* readily formed actively ejected spores after overnight incubation. In contrast, protoplasts treated the same way did not form spores (data not shown). It was possible to infect *T. tabaci* with spores formed on water agar and to reisolate the fungus from the artificially infected thrips (data not shown). Thereby the asexual life cycle of *E. thripidum* under laboratory conditions was completed.

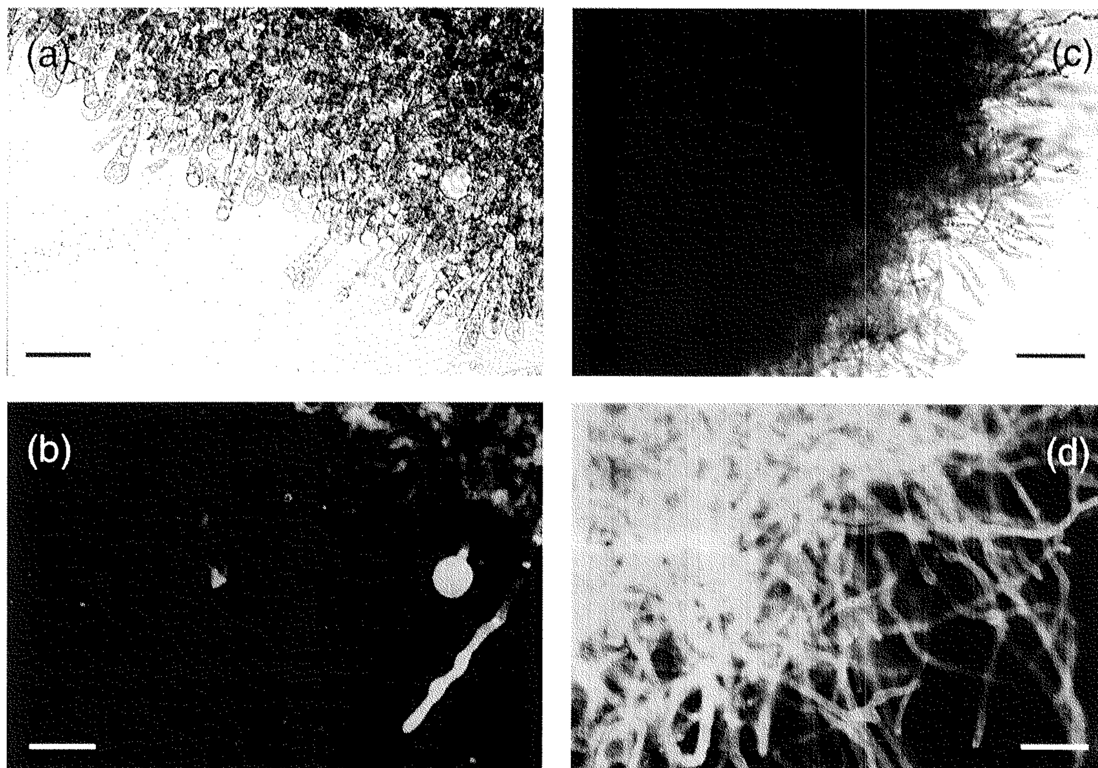


Fig. 1. Light microscopy pictures of *E. thripidum* before (a) and after (c) formation of mycelium. To assess for the presence or absence of a cell wall, cultures were stained with calcofluor and observed under fluorescent light (b and d). A 7 d old culture of *E. thripidum* consisted mainly of protoplasts with only few cells having a cell wall (b). Formation of a cell wall and mycelium occurred within two days (d). Bars a, b, d 50 µm, c 100 µm.

Mycelium formation occurred in stationary phase:-- To understand the signals that led to formation of mycelium cell density, pH, glucose and α-amino acid concentration of the culture medium were followed over a period of 20 d. The *E. thripidum* isolates tested grew exponentially and 10 d after inoculation the cultures had formed mycelium (indicated by arrow in Fig. 2a). At this timepoint the cultures had reached the stationary phase because the density of living cells was decreasing (Fig. 2a). This decline in the measure for the density of living cells was ascribed to lysis of cells within the pellets (Freimoser *et al.*, 2000). The glucose concentration in the culture medium dropped to zero on the 4th day, but increased again between days 6 to 16 (Fig. 2b). It was not clear why glucose concentration was increasing after the 4th day after inoculation (Fig. 2b). It is possible that the lysis of cells within the protoplast pellets led to a release of glucose (Freimoser *et al.*, 2000). Alternatively, substances that interfered with the enzymatic assay used for the determination of glucose were produced by the cultures. However, we did not assess these hypotheses experimentally. The concentration of α-amino acids started to decrease after the 6th day (Fig. 2b), 4 d before the formation of mycelium (at day 10). However, α-amino acids present in the broth were only partially utilised by the fungus (Fig. 2b). The pH of the culture medium behaved similarly to the time course of cell density (Fig. 2c). Starting at 6.5 the pH rose by almost one unit to pH 7.4, before dropping to between 6.8 and 6.9 (Fig. 2c). A rise in pH may be ascribed to the utilisation of amino acids as carbon sources, thereby releasing ammonium. A possible relation of the pH change with the utilisation of amino acids as C-sources was also supported by the coincidence of glucose depletion and pH rise at day 4 (Fig. 2b, c). The reason for the subsequent drop of the pH was not understood, but the production of organic acids after prolonged cultivation could have been responsible for this effect. In contrast to considering the change in pH as a secondary effect it is also possible that the fungus actively changes the pH and that this is used as a signal during pathogenesis, which has been shown for the entomopathogenic fungus *Metarhizium anisopliae* (St. Leger *et al.*, 1999).

Our experiments showed that mycelium formation occurred between days 8 and 10 at the time when the fungus was in stationary phase and suggested that a C- and/or N- limitation was the primary signal leading to induction of mycelium.

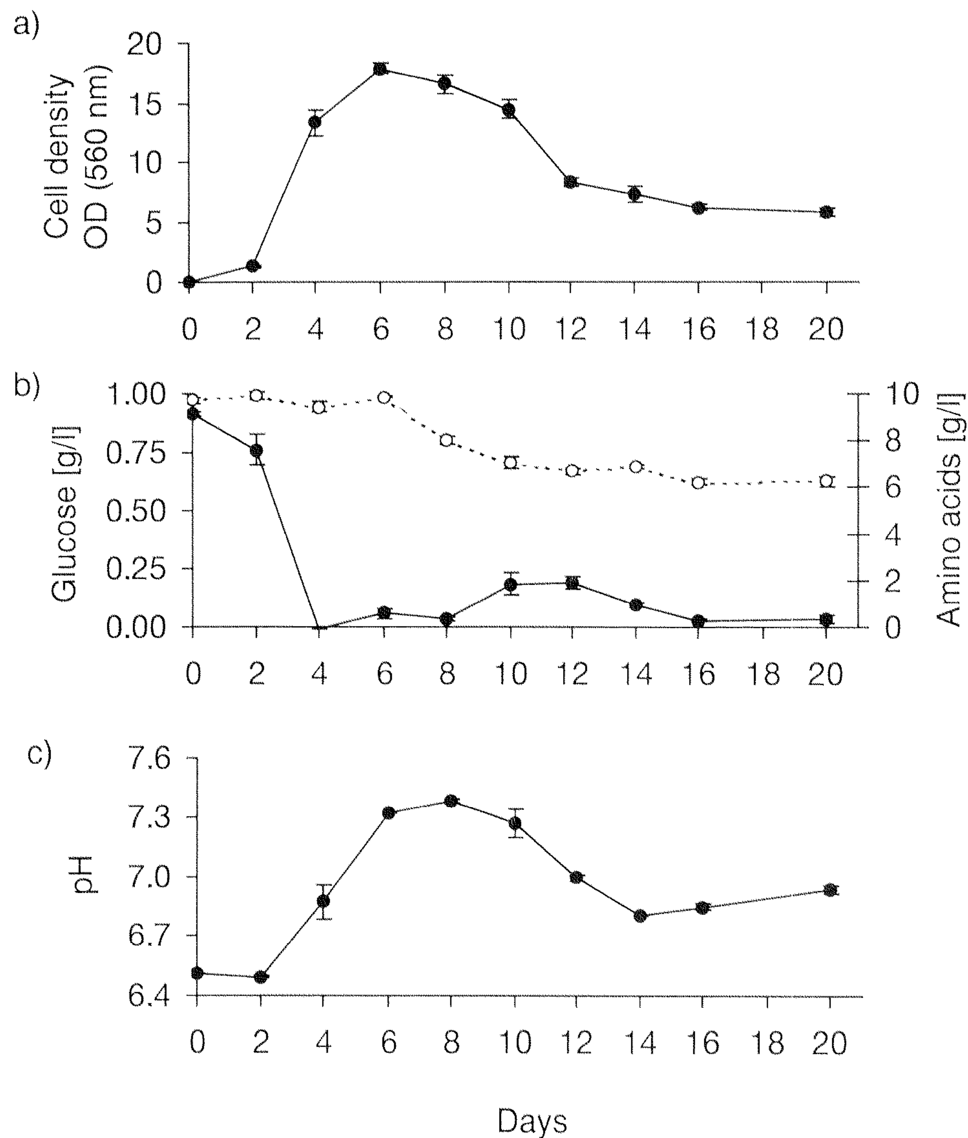


Fig. 2. Time course of an *E. thripidum* culture that formed mycelium spontaneously after 10 days. (a) Change of cell density as measured by the MTT assay over time. (b) Glucose (●) and total α-amino acid (O) measurements in the growth medium. Amino acid concentrations were determined with reference to leucine as a standard. (c) Change of the pH in the medium during growth of *E. thripidum*.

CMM induced differentiation to hyphal growth and formation of mycelium:-- We next tested conditioned medium from different sampling days for the capability to induce mycelium formation. Conditioned medium was taken daily from growing cultures of *E. thripidum* and used to replace the medium from a 7 d old culture. If conditioned medium from a 10 d old culture (CMM; Fig. 3, x-axis) was given to a culture of *E. thripidum* mycelium formation was induced within 2 d (Fig. 3, y-axis). Medium from younger cultures prevented mycelium formation (0-4 d old cultures) or induced differentiation slower (6 and 8 d old cultures,

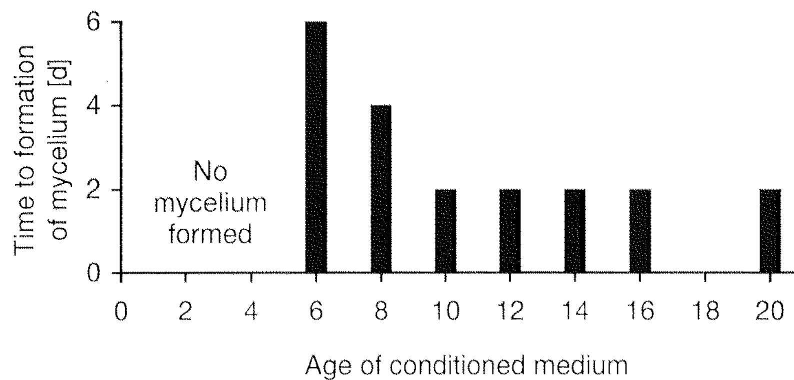


Fig. 3. Conditioned medium from cultures of different age was used to replace half of the culture volume of a 7 days old culture of *E. thripidum*. The age of the culture from which the conditioned medium originated is indicated on the x-axis. In medium from a culture 10 or more days old mycelium had been formed (CMM). The time required to form mycelium after the change of the medium was recorded (y-axis).

Fig. 3). Medium from cultures older than 10 d (CMM) always induced the switch to mycelium within 2 d (Fig. 3). In addition, CMM induced mycelium in a concentration dependent manner and increasing the cell density after 7 d by removing culture medium led to a faster mycelium formation (data not shown). These observations strongly suggested that a change had to occur in the growth medium before mycelium could be formed.

Induction of mycelium by CMM required a responsive stage of E. thripidum*:--* Next, we wanted to test when *E. thripidum* could be induced to form mycelium by the addition of CMM. Therefore an experiment to assess the effect of CMM addition on *E. thripidum* cultures of different ages was performed. CMM was added at different timepoints to a culture and the formation of mycelium was monitored. A culture that was inoculated into medium containing CMM (addition at culture age 0 d) formed mycelium after 8 d (Fig. 4). The time required for the formation of mycelium after the addition of CMM decreased with culture age to only 3 d when a 7 d old culture was induced by CMM addition (Fig. 4). *E. thripidum* cultures older than 7 d consistently formed mycelium within 2-3 d upon CMM addition (Fig. 4). After 15 d the cultures had formed mycelium without the addition of conditioned medium and the experiment was terminated (Fig. 4). These results suggested that the fungus had to grow for 5-7 d (the time point when the curve in Fig. 4 bends) and to reach a certain cell density in order to become responsive to CMM. This experiment also showed that the CMM-induced transformation of protoplasts to the mycelium stage required at least 2-3 d (height of the horizontal part of the curve in Fig. 4). We concluded that

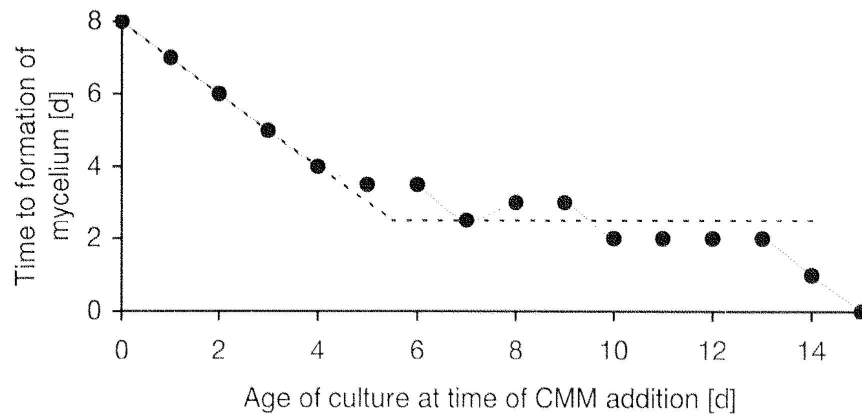


Fig. 4. Competence of mycelium formation at different ages of an *E. thripidum* culture. A culture was grown for 15 d. Every day half of the culture medium was replaced with CMM (x-axis) and the time from the change of the medium to the formation of mycelium was recorded (y-axis).

E. thripidum had to be in a competent state in order to become responsive to CMM. This stage was dependent on the growth phase, again suggesting a control by C- and/or N-limitation in the medium.

Mycelium formation was inhibited by complex nitrogen sources:-- To address the regulatory role of C- and N-limitation in the formation of mycelium, the growth medium of 7 d old *E. thripidum* cultures was replaced with different variations of the GLEN medium. The use of GLEN medium made it possible to omit the C-source (glucose) and/or the N-source (lactalbumin hydrolysate and yeastolate) in the broth. A culture grown in complete GLEN medium formed mycelium after 14 d (7 d after the medium was changed in other cultures, Fig. 5a). However, replacement of the medium after 7 d by complete medium or by medium lacking the C-source suppressed mycelium formation (Fig. 5b, c). Replacement of the growth medium by a medium lacking the N-source, irrespective of the presence or absence of the C-source, induced mycelium formation within 5 d after the addition (Fig. 5 d, e). This was considerably faster than the 7-10 d observed without an exchange of medium (Fig. 5d, e, a respectively). These results showed that mycelium formation was inhibited by adding lactalbumin hydrolysate and yeastolate and suggested that N-limitation was the primary signal for mycelium formation. The effect of CMM in the presence or absence of C- and N- sources was also studied (Fig. 5 f-i). Interestingly, CMM was able to induce mycelium formation in the presence of a C- as well as an N-source. In all cases where CMM was used mycelium was formed after 2-3 days, even if complete GLEN medium was added together with

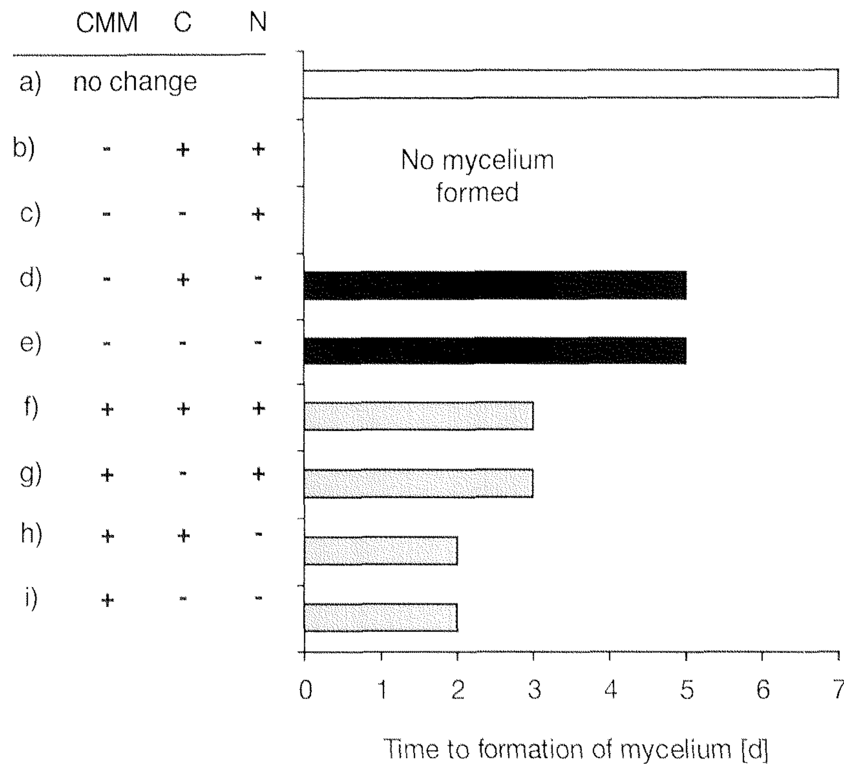


Fig. 5. Mycelium formation in different media. 7 d after inoculation the medium of a culture of *E. thripidum* was left unchanged (a), exchanged with complete fresh medium (b) or with medium lacking the nitrogen and/or the carbon sources (c, d and e as indicated). In addition, complete or depleted fresh medium was used in combination with CMM (f, g, h and i). The x-axis indicates the time elapsed from the moment of medium change to mycelium formation.

the conditioned medium (Fig. 5f, g, h, i). This was substantially faster than the 5 d required for the formation of mycelium in media lacking the nitrogen source (Fig. 5d, e). This indicated that N-limitation was essential to initiate mycelium formation, however, CMM activity was able to bypass this requirement. These results are compatible with the hypothesis that an additional factor, produced and secreted by the fungus under nitrogen limitation, was present in the conditioned medium and induced mycelium formation.

Characterisation of mycelium inducing factor:-- In order to confirm the presence of a mycelium inducing factor and to characterise this activity in CMM, differential extractions, fractionations and protein digestions of CMM were performed. CMM activity was destroyed by boiling, was inactivated by extraction with chloroform and did not pass through filters with 30 kDa cut off size. Thereby it was possible to concentrate CMM activity. Finally, the mycelium inducing activity was abolished by digestion with trypsin. From these results we concluded that the induction of mycelium was caused by a specific activity

present in CMM and our results suggested that a protein larger than 30 kDa was involved in this activity.

DISCUSSION

This report represents the first observation of self-induction of hyphal growth, mycelium formation and subsequent sporulation in entomophthoralean fungi. Based on our results we propose a model for the induction of the mycelium stage in *E. thripidum* where complex nitrogen sources repress the production of a "mycelium-factor" that stimulates the differentiation processes by autoinduction (Fig. 6). Thus, localized N-limitation inside the host insect might result in coordinated mycelium formation of all fungal cells present in the insect. This could provide the mechanism that enables insect pathogenic fungi as *E. thripidum* to sporulate coordinatedly and localised from only a selected spot of the host insect and at a moment when the host is still alive. The time window that allows sporulation from the living and moving insect is very narrow and autoinduction of mycelium formation and subsequent sporulation could provide a mechanism to optimise this process. The selective advantage of sporulation from living and moving hosts might be the spreading of the infectious spores over a larger area, thereby increasing the probability that an infectious spore reaches a new host. Within the framework of this hypothesis it is predicted that entomophthoralean fungi sporulating from cadavers do not require such an autoinduction mechanism, because there is no need for highly organized sporulation. In Entomophthorales that sporulate from dead hosts N-limitation as the primary signal might be sufficient for the direct induction of the sporulation process. In contrast, the regulation of mycelium formation and sporulation via the induction of a "mycelium factor" could represent a general mechanism for fungi sporulating while the host is still alive. Besides for the *E. thripidum* – *T. tabaci* interaction (Samson *et al.*, 1979) sporulation from living hosts is also known for *E. erupta* (Hall, 1959) and for species of the genera *Strongwellsea* and *Massospora* (Strong *et al.*, 1960; White & Lloyd, 1983). It will be interesting to learn if these species also rely on a self-produced factor for the initiation of hyphal growth and subsequent sporulation.

The finding that *E. thripidum* secreted a factor that regulated its own development showed parallels with the quorum sensing phenomenon of bacteria. Autoinduction in bacteria serves as a density-sensing system that controls luminescence, virulence or sporulation (Fuqua *et al.*, 1994; Ji *et al.*, 1995; Denny, 1999; Lazazzera, 2000). This is achieved through an autoinducer synthesised during growth, that accumulates in the surrounding and triggers

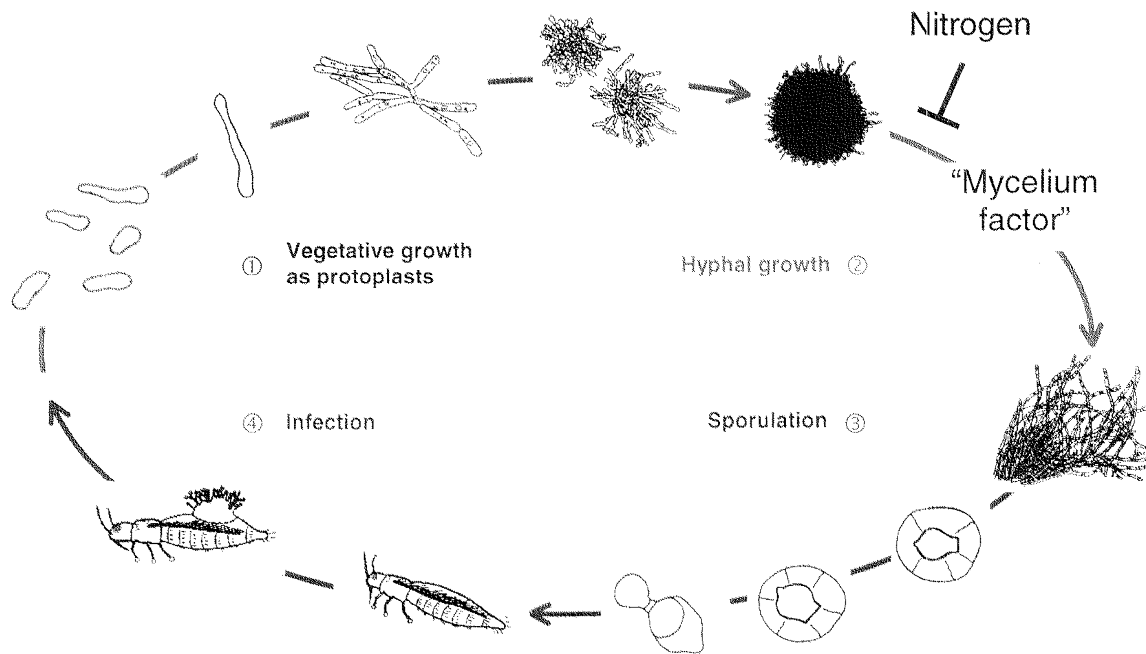


Fig. 6. *In vitro* life cycle and model for the induction of mycelium in *E. thripidum*. Protoplasts grow vegetatively if subcultured regularly (①, blue). These protoplasts produce a "mycelium factor" which induces mycelium formation (②, red). The production of the "mycelium factor" is inhibited by nitrogen. Mycelium is a prerequisite for the formation of actively ejected spores (③, green). These spores are infectious to *Thrips tabaci*. *E. thripidum* can be reisolated from artificially infected thrips thus closing the life cycle under laboratory conditions (④, yellow).

different responses at a threshold concentration (Hastings & Greenberg, 1999). It will be fascinating to elucidate the described mechanism in *E. thripidum* at the molecular and biochemical level to detect possible analogies to the quorum sensing phenomenon of bacteria.

In conclusion the discovery of this regulation mechanism in *E. thripidum* made possible the reliable and controlled production of mycelium and infectious spores, which embodies a first and essential step for a possible utilisation of this fungus as mycoinsecticide. Reliable sporulation also enabled us to infect healthy onion thrips and to complete the asexual life cycle of *E. thripidum* under laboratory conditions (Fig. 6).

ACKNOWLEDGEMENTS

We thank J. Helenius for helpful comment and corrections on the manuscript. This work is supported by the Kommission für Technologie und Innovation (KTI grant 3202.1), and the ETH Zürich.

REFERENCES

Beauvais, A. & Latgé, J. P. (1988). A simple medium for growing entomophthoralean protoplasts. *J Invertebr Pathol* **51**, 175-178.

Brobyn, P. J. & Wilding, N. (1977). Invasive and developmental processes of *Entomophthora* species infecting aphids. *Trans Brit Mycol Soc* **69**, 349-366.

Charnley, A. K. (1989). Mechanisms of fungal pathogenesis in insects. In *The Biotechnology of Fungi for Improving Plant Growth*, pp. 85-125. Edited by Whipps, J. M. & Lumsden, R. D. London: Cambridge Univ. Press.

Denny, T. P. (1999). Autoregulator-dependent control of extracellular polysaccharide production in phytopathogenic bacteria. *European Journal of Plant Pathology* **105**, 417-430.

Evans, H. C. (1989). Mycophathogens of insects of epigeal and aerial habitats. In *Insect-Fungus Interactions*, pp. 205-238. Edited by Wilding, N., Collins, N. M., Hammond, P. M., & Webber, J. F. London: Academic Press.

Freimoser, F. M., Grundschober, A., Aebi, M. & Tuor, U. (2000). *In vitro* cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements, and sporulation. *Mycologia* **92**, 208-215.

Freimoser, F. M., Jacob, C., Tuor, U. & Aebi, M. (1999). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for the colorimetric determination of fungal cell densities. *Appl Environ Microbiol* **65**, 3727-3729.

Frister, H., Meisel, H. & Schlimme, E. (1986). Modifizierte OPA-Methode zur Charakterisierung von Proteolyse-Produkten. *Milchwissenschaft* **41**, 483-487.

Fuqua, W. C., Winans, S. C. & Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269-275.

Grundschober, A. (2000). *In vitro* life cycle of *Neozygites parvispora* and *Entomophthora thripidum*, two fungal pathogens of *Thrips tabaci*. In *Institute of Microbiology*, pp. 127. Zurich: ETH Zurich.

Grundschober, A., Tuor, U. & Aebi, M. (1998). *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *Syst Appl Microbiol* **21**, 461-469.

Hajek, A. E. & St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annu Rev Entomol* **39**, 293-322.

Hall, I. M. (1959). The fungus *Entomophthora erupta* (Dustan) attacking the Black Grass Bug, *Irbisia solani* (Heidemann) (Hemiptera, Miridae), in California. *J Insect Pathol* **1**, 48-51.

Hastings, J. W. & Greenberg, E. P. (1999). Quorum sensing: the explanation of a curious phenomenon reveals a common characteristic of bacteria. *J Bacteriol* **181**, 2667-2668.

Humber, R. A. (1984). Foundations for an evolutionary classification of the Entomophthorales (Zygomycetes). In *Fungus-Insect Relationships: Perspectives In Ecology and Evolution*, pp. 166-183. Edited by Wheeler, Q. & Blackwell, M. New York, N.Y., USA: Columbia University Press.

Ji, G., Beavis, R. C. & Novick, R. P. (1995). Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc Natl Acad Sci USA* **92**, 12055-12059.

Lazazzera, B. A. (2000). Quorum sensing and starvation: signals for entry into stationary phase. *Curr Opin Microbiol* **3**, 177-182.

Lewis, T. (1997). Pest thrips in perspective. In *Thrips as crop pests*, pp. 1-13. Edited by Lewis, T. Wallingford, UK. CAB International.

Samson, R. A., Evans, H. C. & Latgé, J. P. (1988). *Atlas of Entomopathogenic Fungi*: Springer Verlag.

Samson, R. A., Ramakers, P. M. J. & Oswald, T. (1979). *Entomophthora thripidum*, a new fungal pathogen of *Thrips tabaci*. *Can J Botany* **57**, 1317-1323.

St. Leger, R. (1993). Biology and mechanisms of insect-cuticle invasion by Deuteromycete fungal pathogens. In *Parasites and Pathogens of Insects*, pp. 211-229: Academic Press, Inc.

St. Leger, R. J., Nelson, J. O. & Screen, S. E. (1999). The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. *Microbiology* **145**, 2691-2699.

Strong, F. E., Wells, K. & Apple, J. W. (1960). An unidentified fungus parasitic on the seed-corn maggot. *J Econ Entomol* **53**, 478-479.

White, J. & Lloyd, M. (1983). A pathogenic fungus, *Massospora cicadina* (Entomophthorales), in emerging nymphs of periodical cicadas (Homoptera: Cicadidae). *Environ Entomol* **12**, 1245-1252.

APPENDIX: Induction of resting spores in *E. thripidum*

Entomophthorales form thick walled, often darkish coloured spores that serve as a resting stage during unfavourable conditions (Hajek & Humber, 1997; Latgé, 1986). The interest in the formation of resting spores resides in their promise as stable and easily applicable forms of the fungus for the employment as mycoinsecticide (Remaudière, 1971). For many entomophthoralean fungi the formation of resting spores has been observed and environmental conditions fostering their formation have been identified (Glare, *et al.*, 1989; Hajek & Shimazu, 1996; Latgé & Sanglier, 1985; Shimazu, 1979). Resting spores are more likely to form after increased host passages or in later stages of the host insect (Brefeld, 1877; Steinkraus & Kramer, 1989; White & Lloyd, 1983; Wilding & Lauckner, 1974). Other important factors determining the production of resting spores are the duration of *in vitro* culture (Hajek, *et al.*, 1990) or the isolate used (Glare & Milner, 1986; Glare, *et al.*, 1989). But the possibility that the induction of the resting state might be influenced by the host insect or occur without reference to environmental stimuli has also been discussed (MacLeod, *et al.*, 1973). In *E. thripidum* resting spores have not been observed.

In the preceding report the autoinduced differentiation of protoplasts to mycelium was shown to be a prerequisite for sporulation. Among the different isolates of *E. thripidum* ARSEF 5868, 6518, 6521 and 6522 consistently followed this development. Isolate ARSEF 6525 did not differentiate at all and remained as protoplast. The *E. thripidum* isolates ARSEF 6517 and 6519 and sometimes ARSEF 6520 in contrast formed big, vacuolated, spherical cells (Fig. 1a). These cells were also surrounded by a cell wall as shown by calcofluor staining (Fig 1b). In addition to these vacuolated spherical cells, opaque, granular cells were observed in these cultures (Fig. 1c). Consecutively slightly smaller, thick walled, darkish cells appeared in liquid cultures or after transfer onto water agar (Fig. 1d). These cells looked very similar to structures described as resting spores in other Entomophthorales (pictures in Gröner, 1975; Hajek & Humber, 1997; Perry, *et al.*, 1982; Steinkraus & Kramer, 1989).

In analogy we considered the thick walled dark cells as resting spores, while the huge vacuolated cells comprised an intermediate stage in their formation. For *E. thripidum* this is the first observation of such cells. Unfortunately, the resting spores did not germinate or differentiate further making it impossible to prove the biological relevancy of these structures. The formation of spherical cells and resting spores was isolate specific and only observed in isolates ARSEF 6517, 6519 and rarely in 6520. Different samples of the same isolate

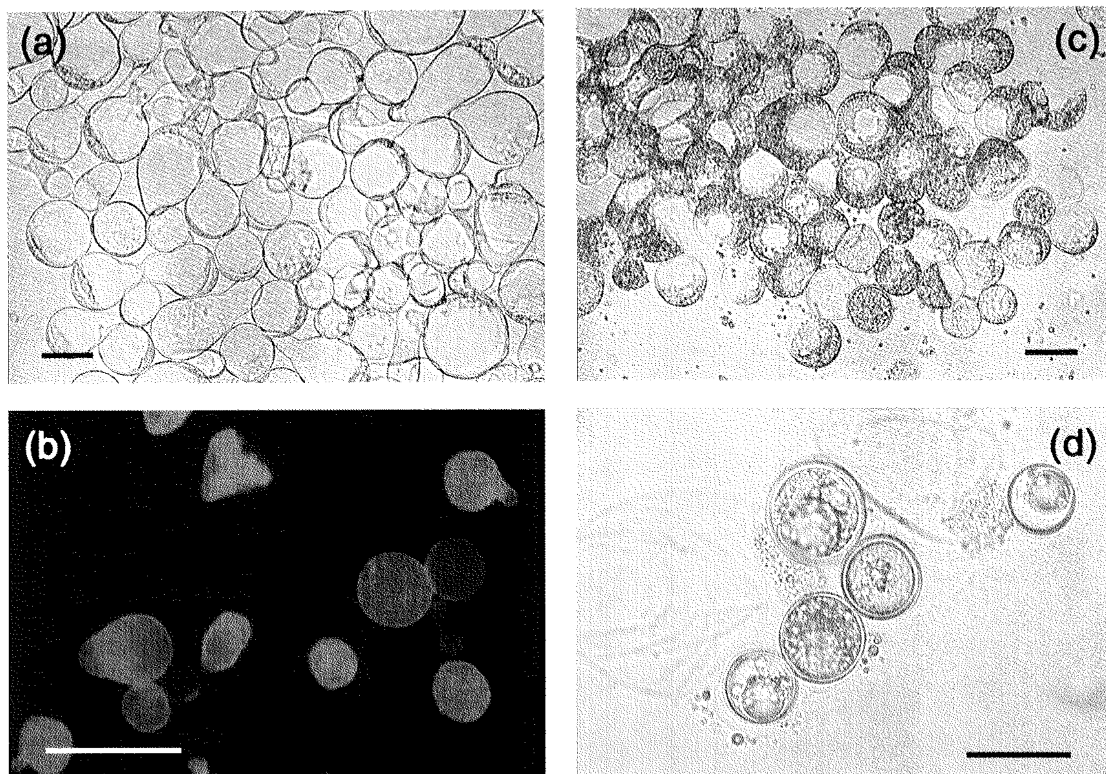


Fig. 1. Different stages of spherical cells and resting spores. (a) Spherical cells with huge vacuoles. (b) Sphericals cells under fluorescent light and stained with calcofluor. The strong coloration indicates the presence of a cell wall. (c) Within the spherical cells granular material formed and filled the cells. (d) Resting spores formed after transfer of spherical cells onto water agar. Bars: a, c, d 20 μm, b 50 μm.

did not all differentiate to spherical cells and resting spores when taken from the freezer; some formed mycelium instead. However, a specific isolate did not switch from mycelium to resting spore formation or vice versa if kept in culture for several months.

Next we tested whether resting spores were induced similarly as mycelium and performed the same experiments as discussed before. As for the switch to mycelium, the formation of resting spores took place after 10 to 20 days in the same culture medium and coincided with a rounding up of all cells and disintegration of the protoplast pellets. The time course for cell density and pH, glucose and amino acid concentrations in the supernatant medium of a spherical cell forming isolate were similar to those of the isolate forming mycelium (data not shown). Conditioned medium of cultures that had formed spherical cells (CMS) also induced spherical cells within 2 to 3 days in a concentration dependent manner. The activity responsible for the formation of spherical cells could be removed from the water phase of CMS by extraction with chloroform. The fraction of CMS most actively and reliably inducing spherical cells also bigger than 30 kDa, but samples containing components smaller than 30 kDa also induced spherical cells sometimes. Neither digestion with trypsin nor heating to 100°C abolished the spherical cell forming activity.

Surprisingly, CMS induced spherical cells in isolates that exclusively formed mycelium and otherwise never formed structures even distantly resembling resting spores or the big vacuolated spherical cells. The reverse, the induction of mycelium with CMM in an isolate that formed spherical cells was not possible. From these results we concluded, that besides the factor inducing mycelium, and thus enabling the formation of infectious spores, there must have been an additional activity that induced the spherical cells and thus lead to resting spores. The spherical cell inducing activity was "dominant" over the mycelium inducing activity as replacing the growth medium of a 7 days old culture with a mixture of CMM and CMS (in ratios 1:2, 1:1 and 2:1) always induced the spherical form of the fungus. Furthermore, in mixed cultures of mycelium and spherical cell forming isolates only the spherical form of *E. thripidum* was observed. In contrast to the differentiation to mycelium the formation of spherical cells and resting spores was not repressed by the addition of complex nitrogen sources.

The observation that the formation of resting spores was isolate dependent is in agreement with reports that show similar phenomena in other entomophthoralean fungi (Glare & Milner, 1986; Glare, *et al.*, 1989; Hajek & Shimazu, 1996). However, in *Entomophaga maimaiga* no resting spore inducing activity was secreted into the culture medium (Kogan & Hajek, 2000). Contrary to these findings the addition of CMS was sufficient for the reliable induction of spherical cells in *E. thripidum*, without changes of temperature, humidity or light regime. In addition, CMS induced spherical cells in *in vitro* cultures of *Entomophthora muscae*, a fungal pathogen of houseflies (not shown). This suggested that self-induction of the resting spore stage through a secreted factor could be a general mechanism in the genus *Entomophthora*. However, the biological function of these resting spores could not be proven and a toxic substance produced by certain isolates of the fungus could have caused the observed development. In this case the formation of resting structures could represent a protective reaction towards harmful metabolites produced by the fungus. It is therefore at present not possible to decide whether the formation of spherical vacuolated cells that led to resting spores represents a regulated development of the fungus. However, the prospect of an autoinduced formation of resting spores represents a fascinating hypothesis for the regulation of the life cycle in entomophthoralean fungi and will require further research.

REFERENCES

Brefeld, O. (1877). Ueber die Entomophtoreen und ihre Verwandten. *Botanische Zeitung* **22**, 345-355.

Glare, T. R., and R. J. Milner (1986). Novel factors affecting resting spore production by *Zoophthora radicans*. In *Fourth International Colloquium of Invertebrate Pathology*, pp. 229. Edited by R. A. Samson, Vlak, J. M., Peters, D. "Konongshof" at Veldhoven, The Netherlands: Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen.

Glare, T. R., R. J. Milner, and G. A. Chilvers (1989). Factors affecting the production of resting spores by *Zoophthora radicans* in the spotted alfalfa aphid, *Therioaphis trifolii* f. *maculata*. *Can J Botany* **67**, 848-855.

Gröner, A. (1975). On resting spores of entomopathogenic Entomophthoraceae (Zygomycetes). 1. Production of resting spores of *Entomophthora thaxteriana* (Petch) Hall et Bell. *Z Pflanzenkr Pflanzenschutz* **82**, 22-29.

Hajek, A. E., and R. A. Humber (1997). Formation and germination of *Entomophaga maimaiga* azygospores. *Can J Botany* **75**, 1739-1747.

Hajek, A. E., R. A. Humber, and M. H. Griggs (1990). Decline in virulence of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) with repeated *in vitro* subculture. *J Invertebr Pathol* **56**, 91-97.

Hajek, A. E., and M. Shimazu (1996). Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth *Lymantria dispar*. *Can J Botany* **74**, 708-715.

Kogan, P. H., and A. E. Hajek (2000). *In vitro* formation of resting spores by the insect pathogenic fungus *Entomophaga maimaiga*. *J Invertebr Pathol* **75**, 193-201.

Latgé, J. P. (1986). The entomophthorales after the resting spore production stage. In *Fundamental and applied aspects of invertebrate pathology*, pp. 651-652. Edited by R. A. Samson, J. M. Vlak, and D. Peters. Wageningen, The Netherlands: Foundation of the Fourth International Colloquium of Invertebrate Pathology.

Latgé, J. P., and J. J. Sanglier (1985). Optimisation de la croissance et de la sporulation de *Conidiobolus obscurus* en milieu defini. *Can J Botany* **63**, 68-85.

MacLeod, D. M., D. Tyrrell, R. S. Soper, and A. J. De Lyzer (1973). *Entomophthora bullata* as a pathogen of *Sarcophaga aldrichi*. *J Invertebr Pathol* **22**, 75-79.

Perry, D. F., D. Tyrrell, and A. J. Delyzer (1982). The mode of germination of *Zoophthora radicans* zygospores. *Mycologia* **74**, 549-554.

Remaudière, G. (1971). Vers l'utilisation pratique des Entomophthorales parasites de pucerons. *Parasitica* **27**, 115-126.

Shimazu, M. (1979). Resting spore formation of *Entomophthora sphaerosperma* Fresenius (Entomophthorales: Entmophthoraceae) (Fungi) in the brown planthopper, *Nilaparvata lugens* (STAL) (Hemiptera: Delphacidae). *Appl Entomol Zool* **14**, 383-388.

Steinkraus, D. C., and J. P. Kramer (1989). Development of resting spores of *Erynia aquatica* (Zygomycetes: Entomophthoraceeae) in *Aedes aegypti* (Diptera: Culicidae). *Environ Entomol* **18**, 1147-1152.

White, J., and M. Lloyd (1983). A pathogenic fungus, *Massospora cicadina* (Entomophthorales), in emerging nymphs of periodical cicadas (Homoptera: Cicadidae). *Environ Entomol* **12**, 1245-1252.

Wilding, N., and F. B. Lauckner (1974). *Entomophthora* infecting wheat bulb fly at Rothamsted, Hertfordshire, 1967-71. *Ann Appl Biol* **76**, 161-170.

CHAPTER 7

Conclusions, opinions and outlook

Seite Leer /
Blank leaf

Conclusions, opinions and outlook

During this PhD work I had the opportunity to work on several aspects of entomophthoralean fungi. By sequencing and phylogenetically analyzing the SSU rDNA (small subunit ribosomal DNA) insight into relationships within the Entomophthorales and with other fungi and even protists was gained. The work on growth requirements of *Neozygites parvispora* and *Entomophthora thripidum* required most of the time, but the results remained inconclusive. Nevertheless, these projects still gave important and interesting indications of the physiology, adaptation to the host, and potential as mycoinsecticides of these entomophthoralean fungi. Finally, the study of differentiation and induction of spore formation in *E. thripidum* pointed to fascinating aspects of regulation in the life cycle of these fungi.

PHYLOGENETIC RELATIONSHIP AND EVOLUTION OF ENTOMOPHTHORALES

Our phylogenetic analysis of SSU rDNA sequences led to the conclusion that microsporidia, obligate intracellular parasites of invertebrates and vertebrates, were Entomophthorales and formed a sister group of Neozygitaceae. These findings prompted us to propose a model where secondary spore formation in Neozygitaceae and spore germination in microsporidia are related processes. This hypothesis could be tested by further analyzing the structure and formation of the capillary tube in Neozygitaceae as well as the process of polar tube formation and emergence in microsporidia. In addition, microsporidia are noteworthy for the structure of the ribosomal RNA and the size of the ribosomes as well as the presence of chitin in spore walls. It would therefore be interesting to assess if the structure of the ribosomal RNA and the sedimentation coefficient of ribosomes of Neozygitaceae support a relationship with microsporidia. Likewise, exact determination of the chemical composition of the microsporidian spore would provide a test for the hypothesis of an entomophthoralean origin of microsporidia. The discovery that microsporidia originated within Entomophthorales also added an additional level of specialization to life strategies exhibited by Entomophthorales. The evolutionary trend within the Entomophthorales leading from saprophytic growth to obligate pathogens and to parasitic organisms is extended to obligate intracellular parasites represented by the microsporidia.

The saprophytic life form and ancestral group within this evolutionary trend in the Entomophthorales is represented by the genus *Conidiobolus*. However, questions about the evolution of the host-pathogen interaction of Entomophthorales with their hosts have not been addressed in the past. It would thus be extremely interesting to use molecular sequence data to estimate when Entomophthorales became pathogenic to arthropods. Thereby the first ancient host of these fungi and the geographic origin of this association could be identified. This would certainly help to explain the geographic distribution and host associations and to better understand the biology of these fungi.

If Entomophthorales had coevolved with their hosts it would be expected that different taxa of host insects are attacked by different taxonomic groups of entomophthoralean pathogens. However, different entomophthoralean families or genera are not strictly associated with defined host groups, suggesting that the Entomophthorales and their host insects did not coevolve. Quite in contrary entomophthoralean fungi are much more prevalent in certain insect taxa and almost completely absent in others. Many host species are attacked by several species of entomophthoralean pathogens even from different genera. Therefore it does not seem that the host species embodied a strong factor for speciation in Entomophthorales. Whatever the reason is for the drive of speciation in entomophthoralean fungi it is clear that different genera use strategies different enough to allow them to share the same hosts. And it is also clear that a better understanding of such phenomena would have important practical value. Interestingly, it was found that two distinct populations of *Metarhizium anisopliae* were not associated with different hosts, but instead with habitat type; one population occurred in an agricultural area whereas the other in forest ecosystems (M. Bidochka and A. Kamp, unpublished results). This example led to the conclusion that not the host species, but the habitat type determines population structure and therefore drive speciation.

GROWTH REQUIREMENTS OF *NEOZYGITES PARVISPORA* AND *ENTOMOPHTHORA THRIPIDUM*

The identification of growth factors for *N. parvispora* and the development of a defined medium for *E. thripidum* was the work into which most of the time was invested and where the least results were obtained. Superficially this leads to the known conclusion that Entomophthorales are extremely fastidious fungi that cannot grow in defined medium without complex supplements. This difficulty to cultivate entomophthoralean pathogens is usually put forward as the main obstacle for their successful employment as biological insecticides. It is of

course true that a fungus requiring FBS or hemolymph is not amenable to large-scale production. But there are several entomophthoralean fungi that grow easily enough to allow production for small-scale glasshouse and field experiments. The reason for the limited success so far with these fungi was not that the pathogens in question were difficult to cultivate. What is much more a problem is the defined production of the desired form of the fungus. And this is rather a biological than a technical problem that requires detailed knowledge about the regulation and induction of different forms of the fungus, as well as an understanding of the interaction with its host.

From the limited experience that I gained with Entomophthorales I conclude that these fungi are so closely adapted to their hosts that they will always require an environment that mimics the incredibly rich enclosure within the insect host. It can not be excluded that Entomophthorales lost the ability to synthesize essential substances like amino acids. Once the fungus overcame the host cuticle and reached the hemocoel it experiences virtually no competition for nutrients. In addition, it is said that one spore of an entomophthoralean fungus is enough to infect an insect. This would mean that within the infected host there is only one individual fungal pathogen. Because there is no competition between different individuals of the same species mutations could accumulate. Since the insect body and especially the hemolymph provide a nutritionally extremely rich environment the loss of the capability to synthesize for example amino acids would not present a major disadvantage. This hypothesis could be tested experimentally by checking for the presence or absence of metabolic functions at the molecular level. Such knowledge could then even be used to engineer strains that can be grown and produced in simpler media.

INDUCTION AND REGULATION OF SPORULATION

The discovery that *E. thripidum* autoinduced sporulation and also resting spore formation was the most fascinating accomplishment of this work. However, this finding just represents a first step and opens the door for many different projects. The most urgent question would of course be the identification of the autoinducing substances. But not only the differentiation, but also the growth as protoplasts is interesting, since it is an exceptional capability among fungi. It would therefore be interesting to know how *E. thripidum* accomplishes to shut off cell wall formation and still to grow. This could then lead to questions addressing cell wall formation that occurs with the switch from vegetative growth to the sporulating stage. This seems especially intriguing to

me because the formation of this cell wall must happen within very short time. I have observed that during growth granules accumulate within protoplasts of *E. thripidum*. These small droplets or vesicles give the cells a darkish appearance and even stain diffusely with calcofluor. I would therefore hypothesize that *E. thripidum* accumulates precursors for cell wall formation in vesicles, which then allow the sudden synthesis of this wall structure. By investigating this process the regulation of growth and sporulation could be elucidated further and *E. thripidum* might even serve as a model for fungal cell wall synthesis in general.

All three projects that I have described here comprise studies on fundamental aspects of the evolution, biology and pathogenesis of these entomophthoralean fungi. But what do the results and the experience gained during this time tell about the potential of biological insecticides based on Entomophthorales? I am persuaded that the major problem for a practical application of this group of fungi is the limited and superficial knowledge about their biology and about "how they work." And I am also convinced that Entomophthorales will become valuable for pest control once they are better understood. Hopefully this study is a contribution to this better understanding and a proof that there is much to be discovered about these fascinating pathogens.

CURRICULUM VITAE

Florian Matthias Freimoser

Born March 10th 1973 in Traunstein, Germany

EDUCATION

1997-2000	PhD study at the Institute of Microbiology, Swiss Federal Institute of Technology, Zurich. **Cultivation, sporulation and phylogenetic analysis of *Neozygites parvispora* and *Entomophthora thripidum*, two fungal pathogens of thrips** Thesis supervisor: Prof. Markus Aebi Group leader: Dr. Urs Tuor
1997	Visit of a field station of Novartis Crop Protection AG in Cairo, Egypt to search for entomopathogenic fungi.
1996	Vistit at ICIPE (International Center for Insect Physiology and Ecology) in Nairobi, Kenya, in the laboratory of Dr. Maniania (microbiology section).
1992-1996	Studies in Systematic and Ecological Biology at the Swiss Federal Institute of Technology Zurich, Switzerland. Final examinations in systematics and taxonomy, applied entomology, mycology, statistics and animal ecology. Diploma thesis in plant ecology under the supervision of Prof. Peter J. Edwards: **Ecosystem development in experimental microcosms**
1986-1992	Secondary school (Kantonsschule Oerlikon, Zurich). Matura of "type C" (mathematics and natural sciences).

"To live, man must hold three things as the supreme and ruling values of his life: Reason - Purpose - self-esteem. Reason, as his ... tool of knowledge - Purpose, as his choice of the happiness which that tool must proceed to achieve - Self-esteem, as his inviolate certainty that his mind is competent to think and his person is worthy of happiness... These three values imply and require all of man's virtues...: rationality, independence, integrity, honesty, justice, productiveness, pride."

Ayn Rand in *Atlas shrugged*

- THE END -