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Entomophthorales on cereal aphids

Characterisation, growth, virulence, epizootiology and potiential for microbial control

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Contents

Preface

This report contains the results of a project conducted at The Royal Veterinary and Agricultural University with financial support from the Danish Environmental Protection Agency. The project was carried out from June 1996 to May 1999.

The steering committee consisted of:

Holger Pedersen, Danish Forest and Nature Agency (Chairman)

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Summary and conclusions

Aphids are important pest insects on cereals in Denmark. The three most important species are: the cereal aphid (*Sitobion avenae*), the bird cherry-oat aphid (*Rhopalosiphum padi*) and the rose-grass aphid (*Metopolophium dirhodum*). Insect-pathogenic fungi from Entomophthorales establish natural epizootics in populations of aphids in cereals. The most common species on aphids on cereals are *Pandora neoaphidis*, *Entomophthora planchoniana* and *Conidiobolus obscurus*.

The project aimed to evaluate the potential of fungi from Entomophthorales for biological control of aphids in cereals, both for direct control ("biopesticides") and for indirect control (enhancing natural epizootics). The report was written based on results obtained in the experimental work and from literature studies.

Morphological, pathobiological and molecular methods were implemented and further developed for characterisation of aphid pathogenic fungi from the genus *Pandora.* It was shown that a correlation between RAPD-PCR profile and the geographical origin of the isolate was present, while no correlation between host species and profile was seen. These methods can be used to study other insect-fungus systems and furthermore for monitoring of isolates released for biological control.

Studies on natural occurrence documented that populations of *R. padi* on the winter host (bird cherry) were infected by a broader range of fungi than in cereals. The species *Neozygites fresenii* and *Zoophthora phalloides* were only found on host insects on their winter host. We consider the interaction between aphid host, summer and winter plant host, and aphids on other host plants as important for the development of epizootics. We found that soil is an important reservoir for winter survival structures of *P. neoaphidis* and *C. obscurus* and infection in spring is likely to begin from these structures.

Successful isolation and growth of *P. neoaphidis* was obtained both *in vivo* and *in vitro.* For small scale experiments *in vivo* cultures are sufficient, while *in vitro* propagation is necessary for experiments where large amounts of inoculum are needed (e.g. for field releases). The *in vitro* growth experiments focused mainly on the importance of agitation speed. High virulence of *P. neoaphidis* against *S. avenae* and *R. padi* was documented in bioassays. Calculated LC_{50} revealed that alates were more susceptible than apterae. LT_{50} for *P. neoaphidis* infected individuals was strongly dependent on incubation temperature.

A biological conceptual model was created to encourage a deeper understanding of a system consisting of *S. avenae* and *P. neoaphidis*. Our experimental data and data from the literature were used to develop the model, which then pointed out factors of significance in the development of epizootics.

It can be concluded that fungi from Entomophthorales possess great potential for future, biological control of aphids in cereals and other crops. Possible strategies include developing the fungi as biopesticides or using them to enhance natural epizootics.

It can be confirmed that particularly *P. neoaphidis* has potential as a future biopesticide. Further development depends on additional experiments, including technical studies involving production and formulation. Nevertheless documentation of the effects following outdoor release is still required.

The potential of a strategy for enhancing natural epizootics was also supported in the project. In particular, the importance of alternative aphid hosts, winter host plants for aphids and the soil was elucidated. Additional studies including factors we now hypothesise are important, e.g. landscape elements, are necessary before an operational strategy is possible.

Sammenfatning og konklusioner

Bladlus er betydende skadedyr på kornafgrøder i Danmark. De tre vigtigste arter af bladlus på korn er: Kornbladlus (*Sitobion avenae*), havrebladlus (*Rhopalosiphum padi*) og græsbladlus (*Metopolophium dirhodum*). Insektpatogene svampe fra Entomophthorales danner naturlige epidemier i populationer af bladlus i korn. De almindeligste arter på bladlus i korn er: *Pandora neoaphidis*, *Entomophthora planchoniana* og *Conidiobolus obscurus*

Projektet sigtede mod at afklare potentialet af svampe fra Entomophthorales til biologisk bekæmpelse af bladlus i korn, både som direkte bekæmpelsesmidler ("biopesticider") og ved indirekte bekæmpelse (understøttelse af naturlig epidemier). Rapporten er skrevet udfra de opnåede resultater samt litteraturstudier af emnet.

Morfologiske, patobiologiske og molekylærbiologiske metodesæt blev anvendt og videreudviklet til karakterisering af bladluspatogene svampe fra slægten *Pandora*. Det kunne påvises, at der var sammenhæng mellem RAPD-PCR data og geografisk oprindelse af isolater, mens der ikke var sammenhæng mellem profilerne og insektart. Metodesættet indarbejdet under dette projekt kan anvendes til andre insekt-svampe systemer samt til monitering af isolater, der er udbragt til biologisk bekæmpelse.

Undersøgelser af den naturlige forekomst viste, at havrebladlus på vinterværten hæg angribes af et bredere spektrum af svampe end i korn. Således kunne arterne *Neozygites fresenii* og *Zoophthora phalloides* kun påvises på værtinsektet på vinterværten. Samspil mellem bladlus, sommer- og vintervært, bladlusarter på andre værtplanter vurderes som vigtige for udvikling af epidemier. Endvidere blev det vist, at jordbunden er et vigtigt reservoir for de overlevelsesstrukturer af *P. neoaphidis* og *C. obscurus*, der starter infektion om foråret.

I projektet blev *P. neoaphidis* isoleret og dyrket både *in vivo* og *in vitro*. Dyrkning *in vivo* er tilstrækkelig til mindre laboratorieforsøg, mens *in vitro* opformering er nødvendig, hvis større mængder inokulum skal anvendes eksempelvis ved udbringning i marken. Der blev udført forsøg med *in vitro* kulturer af *P. neoaphidis* i flydende vækstmedier med fokus på betydningen af omrøringshastigheden. En række bioassay forsøg dokumenterede høj virulens af *P. neoaphidis* overfor korn- og havrebladlus. De beregnede LC_{50} værdier viste, at vingede kornbladlus var mere modtagelige end uvingede individer. LT_{50} for bladlus inficeret med *P. neoaphidis* var stærkt temperaturafhængig.

En biologisk konceptuel model blev opstillet med henblik på en bedre forståelse af et system bestående af *S. avenae* og *P. neoaphidis*. Data fra vores eksperimenter samt data fra litteraturen indgik i denne model. Modellen påpegede en række faktorer, der påvirker epidemiudviklingen i populationer af bladlus.

Samlet kan det konkluderes, at svampe fra Entomophthorales har et højt potentiale som en del af en fremtidig, biologisk bekæmpelse af bladlus i korn og andre afgrøder. Strategien kan både være, at udvikle svampene som biopesticider til direkte bekæmpelse eller at understøtte de naturligt forekommende epidemier.

Projektets data kan bekræfte, at især *P. neoaphidis* har potentiale som biopesticid. Videreudvikling vil kræve en række yderligere forsøg, tildels af teknisk karakter. Der kræves således forbedrede *in vitro* produktions og formuleringsmetoder. Men der kræves også en dokumentation af, at svampen virker efter udbringning på friland.

Potentialet for en strategi med understøttelse af naturlige epidemier kan også bekræftes af projektets data. Især er betydningen af alternative bladlusværter, bladlusenes vinterværter og jordbunden klarlagt. Der kræves dog også her supplerende undersøgelser, før en samlet strategi med inddragelse af f.eks. landskabelige elementer til støtte for naturlig bekæmpelse af bladlus i korn kan udarbejdes.

1 Introduction

1.1 **Background**

Cereal aphids

Aphids (Homoptera: Aphididae) are among the most important pest insects in agriculture in the temperate climatic zones (Minks & Harewijn, 1988). Although more than 40 species of aphids are associated with cereal (Vickerman & Wratten, 1979), only three species are of economic importance in Denmark: the English grain aphid *Sitobion avenae* (F.), the bird cherry-oat aphid *Rhopalosiphum padi* (L.) and the rose-grass aphid *Metopolophium dirhodum* (Walk.). In this report the three common species are designated cereal aphids.

Life cycle

Cereal aphids are holocyclic, which means that during a year both agame and gamogane generations occur. *S. avenae* are monoecious on cereal and grasses (Gramineae), and *R. padi* and *M. dirhodum* are heteroecious between bird cherry (*Prunus padus*) and Gramineae in the first case and roses and Gramineae in the second case (Vickerman & Wratten, 1979). All species overwinter in the egg stage and in spring fundatrices hatch from eggs and later they start to produce parthenogenetic offspring. For the two heteroecious species *R. padi* and *M. dirhodum,* an increasing number of individuals will develop wings and usually after two to three generations they will migrate from their primary (winter) to their secondary (summer) host (Dixon, 1973; Vickerman & Wratten, 1979; Hansen, 1995).

The emigrants colonise Gramineae on which several generations are produced (Dixon, 1973, Hansen 1995). On wheat, *S. avenae* prefers the upper leaves and then ears once these have emerged, *M. dirhodum* feeds on the leaf, and *R. padi* feeds primarily near the leaf bases and behind the leaf sheets (Dean, 1974). The relative abundance of the three species varies in both space and time (Hansen, 1995). When aphids are overcrowded, alate individuals develop and fly off to colonise other grass plants (Dixon, 1973; Vickerman & Wratten, 1979).

In autumn, alate gynoparae and alate males are produced in response to short day length and low temperature, and the gynoparae are always produced before the males (Dixon & Glen, 1971). *R. padi* and *M. dirhodum* fly to their primary host, while *S. avenae* remains on grasses where the gynoparae give birth to oviparae. After mating, the oviparous females lay the overwintering eggs (Dixon, 1973; Vickerman & Wratten, 1979; Hansen, 1995).

Natural enemies

A range of natural enemies such as predators, parasitoids and pathogens regulates aphid populations in cereal fields. Among the pathogens, all of the most prevalent and widely encountered species belong to the order Entomophthorales (Dean & Wilding, 1973; Dedryver, 1983; Feng *et al*., 1991). The species of entomophthoralean fungi identified from aphids belong to five genera: *Conidiobolus, Entomophthora, Pandora, Neozygites* and *Zoophthora* (Latgé & Papierok, 1988).

Epizootics caused by these fungi are often observed in cereals (Dean & Wilding, 1973; Dedryver, 1983; Feng *et al*., 1991; Steenberg & Eilenberg, 1995). Prevalence of infection may in some periods exceed 80%, indicating the possibility of utilising entomophthoralean fungi in microbial control of aphids either by developing a strategy for augmentation based on one of the

fungi or by manipuling the environment so that the natural occurrence of fungi is favoured. Before augmentation or manipulation can be realized however, a better understanding of the epizootiology is necessary. However, most attention so far has been given to the effects of entomophthoralean fungi in cereal crops during the summer months. Thus knowledge concerning their effects during winter, spring and autumn and the possible interactions with other ecosystems is very limited.

1.2 **Life cycle of Entomophthorales**

Entomophthorales

The order Entomophthorales belongs to the subdivision Zygomycotina in the class Zygomycetes. Most Entomophthorales are pathogens to insects, however a few species are also saprophytes in soil.

Life cycle

In the aphid system all Entomophthorales have the same overall pattern of life cycle (figure 1.1). From the infected aphids, primary conidia are forcibly discharged. Primary conidia produce secondary conidia, which like the primary conidia are either forcibly discharged or are produced on long, slender conidiophores. Once the conidia land on a susceptible host under favourable conditions they will produce a germ tube that directly penetrates the insect cuticule (or first produce an appressorium and then penetrate the cuticle). Both enzymatic and physical processes are involved in the penetration. Once the fungus has penetrated the cuticle it will start to multiply and, after a period, the fungus will have invaded all the host tissues and the insect dies. The life cycle of the fungus can then follow one of two paths (1): An asexual path where conidiophores emerge through the insect integument and conidia are formed, or (2): A path where zygo- or azygospores (resting spores) are formed (Tanada & Kaya, 1993).

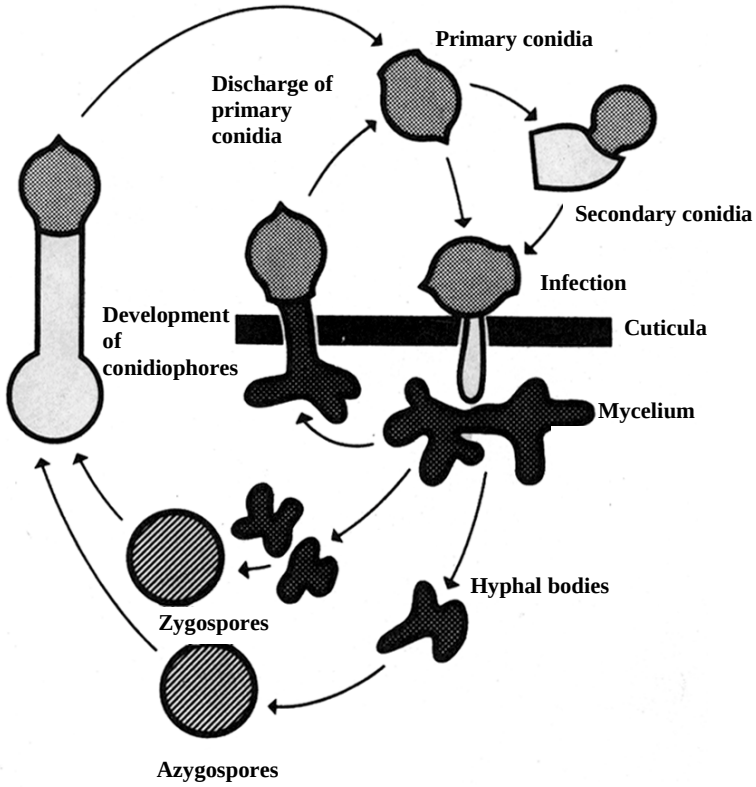

Figure 1.1
Generalised life cycle of Entomophthorales (modified after Eilenberg, 1983).

Epizootiology

Before infection of an aphid can occur, it is necessary with contact between the infective unit and the aphid. Tanada & Kaya (1993) reported that the spread of disease depends on both densities of the host and the infective unit. An increase in one or both pools enhances the probability for contact between conidia and aphids. In addition to the contact between conidia and aphids, suitable temperatures and relative humidities are necessary for sporulation, germination of conidia and for penetration of the aphid integument (Benz, 1987). Humidity and precipitation are often mentioned particularly as key factors (Missonnier *et al.*, 1970; Dean & Wilding, 1971, 1973; Wilding 1975; Dedryver, 1983; Benz, 1987).

1.3 **Project objectives**

Project aim

The aim of the project was to clarify the potential of insect pathogenic fungi for better microbial control of cereal aphids.

Emphasis was on fungi belonging to the order Entomophthorales, particularly *Pandora neoaphidis* (Remaudière & Hennebert) Humber and *S. avenae* and *R. padi*. A number of biological parameters have been investigated to assess which control strategy has the greatest potential. This report contains both the results of our experimental work and extracts from the literature.

1.3.1 **Specific project objectives**

Specific objectives

The specific objectives of this project were as follows:

- To implement and develop morphological, pathobiological and molecular characterisation methodologies for *P. neoaphidis* (chapter 2)
- To describe the natural occurrence of Entomophthorales in different ecosystems relevant to cereal aphids (chapter 3)
- To investigate the winter survival of entomophthoralean fungi infecting cereal aphids (chapter 4)
- To implement and develop methods for *in vivo* and *in vitro* isolation and growth of Entomophthorales with emphasis on *P. neoaphidis* (chapter 5)
- To investigate the virulence of *P. neoaphidis* against *R. padi* and *S. avenae* (chapter 6)
- To describe the interactions between *P. neoaphidis* and cereal aphids, exemplified by a biological conceptual model of a system consisting of *P. neoaphidis* and *S. avenae* (chapter 7)
- To evaluate the potential of Entomophthorales for controling aphids in cereals (chapter 8)

2 Characterisation of Entomophthorales

The order Entomophthorales

The order Entomophthorales (subdivision: Zygomycotina) is characterised by a sporangium that has been reduced to a single conidium and is usually discharged forcibly at maturity. Primary conidia may produce secondary conidia. Fungi from Entomophthorales produce thick-walled resting spores (zygospores or azygospores). Most species are parasitic on insects and in a few cases on other animals and plants, or they live saprophytically in soil and dung (Webster, 1970; Humber, 1989; Tanada & Kaya, 1993). The mycelium of Entomophthorales is coenocytic, however it may become divided by septae into segments. Somatic protoplasts are common in the order. In some species the mycelium fragments into hyphal bodies (Alexopoulos *et al.*, 1996). Generally the characterisation of Entomophthorales into families, genera and species is based on classical mycological features.

2.1 Characterisation by morphological and pathobiological methods

Families

Within the order Entomophthorales, family characteristics include nuclear cytology: relative nuclear size and appearance and the quantity and distribution of condensed chromatin during interphase, morphology of mitotic chromosomes, the relative placement of the spindle at metaphase and the fate of the nuclear envelope during mitosis (Humber, 1989). Knowledge concerning the modes of formation and germination of resting spores and the nature of vegetative growth and development is also used to separate families (Humber, 1989). The following five families are recognised within the Entomophthorales: Completoriaceae (Humber), Meristacraceae (Humber), Ancylistaceae (Fisher), Entomophthoraceae (Winter) and Neozygitaceae (Ben-Ze'ev & Kenneth). Only the latter three contain entomopathogens (Humber, 1989; Keller, 1999).

Genera

The classification of genera in the Entomophthorales has over the years undergone revision (McCoy *et al.*, 1988). At present primarily three systems are normally used, referred to as Humber's classification, Keller's classification and Bałazy's classification. According to Keller (1991; 1994) the following characters should be used to define the genera: mode of discharge of primary conidia, number of nuclei per conidium, shape of primary and secondary conidia, mode of formation of secondary conidia and finally pathobiology (e.g. host symptoms). Humber (1989) further focused on the nature of the conidial wall and morphology of the primary conidiophores and / or conidiogenous cells but not the morphology of the secondary conidia and the pathobiology. However, Humber (1989) includes the presence and morphology of cystidia and rhizoids, types of secondary conidia formed and the pathobiology if these are correlated to characteristics of the primary conidia. Bałazy (1993) agrees with Keller (1991; 1994) and Humber (1989) concerning most points, but includes *Neozygites* in Entomophthoraceae and includes all species with one nucleus per conidium in the genus *Zoophthora.* The current classifications for entomopathogens within Entomophthorales are shown in table 2.1. In this report the system proposed by Humber (1989) will be cited. Humber's features for classification are illustrated in figure 2.1.

Table 2.1
Classification of entomopathogenic fungi within the order Entomophthorales (Bałazy, 1993; Remaudière & Hennebert, 1980; Remaudière & Keller, 1980; Keller, 1987, 1991, 1999; Humber, 1981, 1989; Steinkraus et al., 1998).

Bałazy's classification	Keller's classification	Humber's classification
Ancylistaceae	Ancylistaceae	Ancylistaceae
Conidiobolus	*Conidiobolus*	*Conidiobolus*
Entomophthoraceae	Entomophthoraceae	Entomophthoraceae
Entomophaga	*Entomophaga*	*Entomophaga*
Batkoa	*Batkoa*	*Batkoa*
Entomophthora	*Entomophthora*	*Entomophthora*
Massospora	*Massospora*	*Massospora*
Zoophthora	③	③
subg. *Zoophthora*	*Zoophthora*	*Zoophthora*
subg. *Neopandora*	*Erynia*	*Pandora*
subg. *Erynia*	*Erynia*	*Erynia*
subg. *Furia*	*Erynia*	*Furia*
Strongwellsea	*Strongwellsea*	*Strongwellsea*
Eryniopsis	*Eryniopsis*	*Eryniopsis*
①	*Orthomyces*	*Orthomyces*
②	Neozygitaceae	Neozygitaceae
Neozygites	*Neozygites*	*Neozygites*

① New genus described in 1998 (Steinkraus *et al*., 1998).
② Bałazy considers *Neozygites* to be part of Entomophthoraceae.
③ Keller and Humber do not use *Zoophthora* as a genus name for all uninucleate genera.

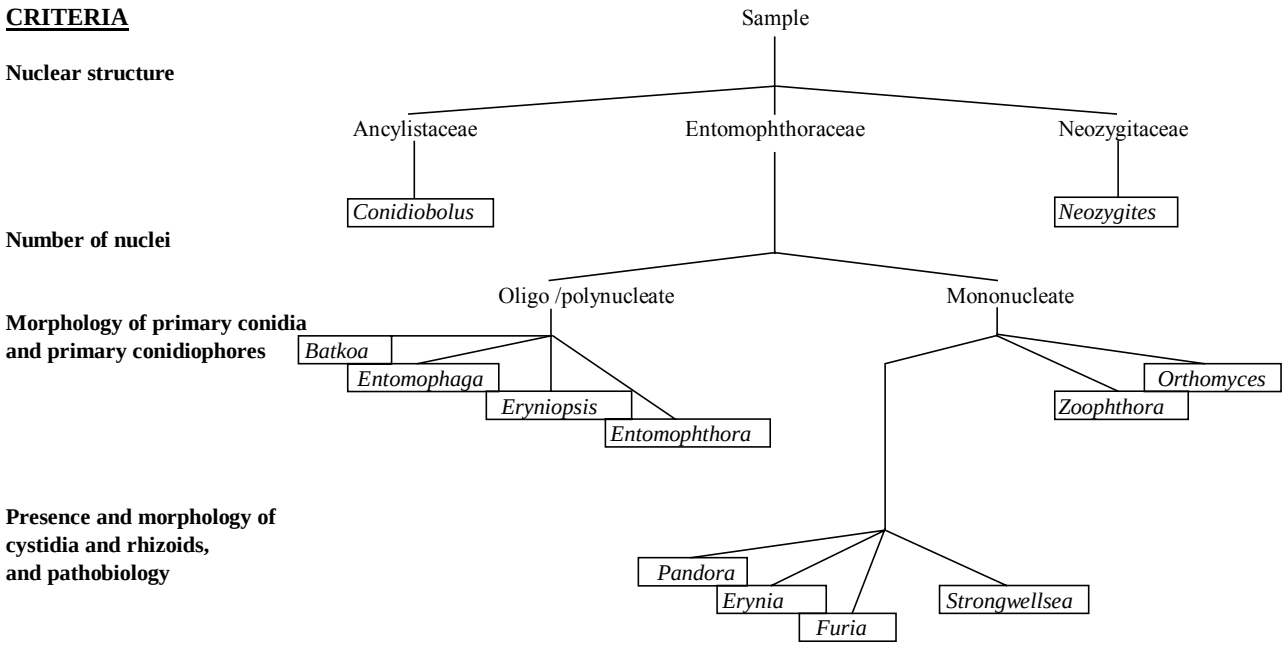

Figure 2.1

Key for identification of entomopathogenic Entomophthorales to genus level following Humber's system (Humber, 1989, 1997). (Figure modified after Keller, 1994).

Species

Species are identified on the basis of host insect species and morphological features, primarily the size and shape of the primary conidia and the number of nuclei per conidium. Keys for identification of species are given by Bałazy (1993). Keys for the species found in Central Europe are given by Keller (1987, 1991). A key to all species of *Neozygites* is given by Keller (1997b) and an overview of all species of *Eryniopsis* is given by Keller & Eilenberg (1993). Finally an overview of the *Entomophthora muscae* (Cohn) Fresenius complex is given by Keller *et al.* (1999). An overview of morphological features for species infecting aphids is given in Appendix A.

Strain and isolate

Characterisation methods based on morphology and pathobiology are insufficient to distinguish between different strains and isolates of the same species. However, for studies on the development of epizootics it is essential to differentiate between strains. For monitoring the establishment and spread of a released fungus it is essential to identify the released isolate from naturally occurring strains. Methods based on either biochemical reactions or DNA are therefore used for that purpose. In this report the term 'strain' is used for a group of clonally related species and the term 'isolate' is used for the culture itself (Hawksworth *et al.*, 1995).

2.2 Characterisation by biochemical methods

Biochemical data have been used to assess intraspecific and interspecific variation amongst isolates. The most used methods are electrophoretic mobility of enzymes and isozymes as well as fatty acid composition (May *et al.*, 1979; Milner *et al.,* 1983; Latgé & Boucias, 1984; Glare *et al.*, 1987; Wilding *et al.*, 1993).

Interspecific variation

In general, gel electrophoresis of enzymes and fatty acid compostion are suitable for distinguishing between species though not between isolates of the same species (May *et al.,* 1979; Milner *et al.,* 1983; Glare *et al.* 1987; Wilding *et al.* 1993). As an example Wilding *et al.* (1993) identified entomophthoralean fungi in aphid hosts to species level using gel electrophoresis of enzymes. They found that among the thirteen tested enzymes three of them were able to distinguish between the two aphid pathogens *P. neoaphidis* and *Conidiobolus obscurus* (Hall et Dunn) Remaudière & Keller but not between isolates of the same species.

Intraspecific variation

Electrophoresis of isozymes is in contrast useful for investigating intraspecific variation between species. Latgé & Boucias (1984) examined the intraspecific variation of *C. obscurus* using electrophoresis of isozymes. They demonstrated that isoenzyme banding patterns were independent of host species, geographical origin, mycelia age and culture medium among 30 isolates of *C. obscurus.* However, the isolates allowed a clustering based on their ability to produce resting spores, sporulate and infect aphids. Similarly, Silvie *et al.* (1990) demonstrated using isoenzyme analysis that a strain of *P. neoaphidis* released in a greenhouse experiment for controlling aphids was gradually replaced by naturally occurring strains of the same fungus species.

2.3 **Characterisation by DNA based methods**

The characterisation of fungi using DNA based techniques has during the last ten years become increasingly important. The methods are used for studies in evolutionary ecology, population genetics and systematics. DNA based methods has several significant advantages over alternatives such as morphological and biochemical characterisation because the genotype rather than the phenotype is assayed (Dowling *et al.*, 1996). Furthermore, some methods are able to distinguish between isolates of the same species, and methods can be applied based on analysis of only a small amount of tissue.

PCR

Over the years different polymerase chain reaction (PCR) based methods have been developed. In table 2.2 some of the commonly used techniques and their applicability to different problems relevant to entomopathogenic fungi are listed.

Table 2.2
*Commonly used PCR based techniques and their applicability to different problems in characterisation. Techniques marked with * have been used for Entomophthorales (Hodge* et al., *1995; Thomsen & Beauvais, 1995; Dowling* et al., *1996*; *Hajek* et al., *1996; Jensen* et al., *1998*; *Rohel* et al., *1997; Jensen* et al., in press; *Vestergaard & Eilenberg*, in press).

Assays / *Application*	**Sequencing***	**RFLP***	**SSCP DGGE**	**AFLP**	**RAPD***	**UP-PCR**
	← Specific priming →				← Random priming →	
Finger printing	-	+	++	++	++	++
Diversity	(+)	+++	++	+	+	+
Relatedness	++	++	++	-	-	-
Geographical variation	-	+++	++	++	++	++
Phylogeny	+++	+	-	-	-	-

RFLP: Restriction fragment length polymorphism ; SSCP: Single-strand conformation polymorphism; DGGE: Denaturing gradient gel electrophoresis; AFLP: Amplified fragment length polymorphism; RAPD: Random amplified polymorphic DNA; UP-PCR: Universal priming – polymerase chain reaction.

2.3.1 **Molecular and morphological variation in *Pandora neoaphidis***

Objective

The objective of our studies was to obtain information about the diversity of *P. neoaphidis* isolated from different hosts and geographical origins using both classical characterisation methods (conidial morphology) and molecular techniques (PCR).

RAPD

Random amplified polymophic DNA-PCR (RAPD) was the technique chosen for analysing the geographical variation among *P. neoaphidis* isolates. As can be seen in table 2.2, RAPD is one of the techniques that is useful for studies of geographical variation.

RAPD-PCR analysis involves amplification of random segments of genomic DNA. Usually 10 base oligonucleotide primers are used to amplify the DNA. A DNA amplification product is generated for each genomic region that happens to be flanked by a pair of 10 base priming sites. The DNA fragments generated in the RAPD reactions are separated electrophoretically on an agarose gel and visualised by ethidium bromide staining. Under standardised conditions, individuals of the same genotype can be expected to show identical RAPD fragment profiles that are likely to differ from those of

other genotypes. The protocol used in our experiments is included in appendix B.

Morphology

Length and width of conidia were measured.

Isolates

Sixteen isolates of *P. neoaphidis* were selected from the ARSEF collection of entomopathogenic fungi. The isolates were selected to cover as many geographical regions in the world as possible. Isolates from either *Acyrthosiphon pisum* (Harr.), *Brevicoryne brassicae* (L.) or *S. avenae* were chosen primarily.

For comparison, other species of *Pandora* isolated from aphids were selected for the study (*Pandora nouryi* (Remaudière & Hennebert) Humber and *Pandora kondoiensis* (Milner in Milner, Mahon & Brown) Humber). In addition two species of *Pandora* isolated from other insect families were included (*Pandora bullata* (Taxter & MacLeod in Humber) Humber and *Pandora delphacis* (Hori) Humber). Finally two isolates from the genus *Conidiobolus* isolated from aphids were included as outgroups. A list of the isolates included in our study is given in table 2.3.

Table 2.3

Insect host and geographic origin of isolates used for RAPD analyses. Isolates written in bold and italics were additionally used for morphological studies.

Species and ARSEF or KVL accession number	Host	Collection site	Year
Pandora neoaphidis			
639	*Aphis fabae* (Hem.: Aphididae)	Poland: Szczecin	1981
827	*Acyrthosiphon pisum* (Hem.: Aphididae)	Australia: New South Wales	1982
833	*Hyperomyzus lactucae* (Hem.: Aphididae)	Chile: Easter Island	1982
1598	*Acyrthosiphon pisum* (Hem.: Aphididae)	France: Avignon	1984
1603	*Acyrthosiphon pisum* (Hem.: Aphididae)	Spain: St. Amalia	1984
1617	*Brevicoryne brassicae*(Hem.: Aphididae)	France: La Minière	1984
1985	*Aphis fabae* (Hem.: Aphididae)	France: La Minière	1985
2018	*Acyrthosiphon pisum* (Hem.: Aphididae)	Yugoslavia: Voyvodina	1985
2583	*Acyrthosiphon pisum* (Hem.: Aphididae)	USA: Lansing, New York	1988
3237	*Acyrthosiphon pisum* (Hem.: Aphididae)	USA: Vernon, Washington	1991
3240	*Acyrthosiphon pisum* (Hem.: Aphididae)	USA: Moscow, Idaho	1991
3241	*Aphis fabae* (Hem.: Aphididae)	USA: Yakima, Washington	1991
5372	*Brevicoryne brassicae*(Hem.: Aphididae)	Denmark: Hegnstrup	1996
5374	*Brevicoryne brassicae*(Hem.: Aphididae)	Denmark: Hegnstrup	1996
5403	*Sitobion avenae* (Hem.: Aphididae)	Denmark: Copenhagen	1995
KVL 630	*Sitobion avenae* (Hem.: Aphididae)	Denmark: Ågerup	1995
Pandora kondoiensis			
825	*Acyrthosiphon kondoi* (Hem.: Aphididae)	Australia: Queensland	1977
828	*Acyrthosiphon kondoi* (Hem.: Aphididae)	Australia: New South Wales	1980
836	*Acyrthosiphon kondoi* (Hem.: Aphididae)	Australia: New South Wales	1981
Pandora nouryi			
199	Unknown (Hem.: Aphididae)	USA: Stillwater, Maine	1977
362	*Acyrthosiphon kondoi* (Hem.: Aphididae)	Australia: Queensland	1979
Pandora delphasis			
134	*Nilaparvata lugens* (Hem.: Delphacidae)	Japan: Fukuoka	1975
478	*Nephotettix cincticeps* (Hem.: Cicadellidae)	Phillipines: Manila	1980
580	*Nilaparvata lugens* (Hem.: Delphacidae)	Indonesia: Sumatra	1981
716	*Nilaparvata lugens* (Hem.: Delphacidae)	China: Jiangsu	1981
1936	Unknown (Hem.: Cicadellidae)	Brazil: Goiás	1985
3107	*Empoasca fabae* (Hem.: Cicadellidae)	USA: Catherine, New York	1990
Pandora bullata			
116	*Phaenicia sericata* (Diptera: Calliphoridae)	USA: Eastham, Massachusetts	1972
Conidiobolus obscurus			
3141	*Acyrthosiphon pisum* (Hem.: Aphididae)	USA: California	1972
Conidiobolus thromboides			
115	*Therioaphis maculata* (Hem.: Aphididae)	USA: Catherine, New York	1990

Results

RAPD-PCR was implemented and further developed for this system. Ten out of fourteen primers amplified multiple DNA fragments for all isolates included in this study. A total of 568 discrete bands were scored from photographs using the ten primers. Phenetic similarity was calculated (UPGMA using Jaccard's coefficient) by using the statistical software NTSYSpc (V.2.01e). The tree generated by the analysis is shown in figure 2.2. All *P. neoaphidis* isolates shared more than 40% of the scored bands while sharing virtually none with other species, even other *Pandora* species isolated from aphids. Among the *P. neoaphidis* isolates, RAPD grouping could be related to geographical origin. No relationship between host insect and RAPD groups was seen.

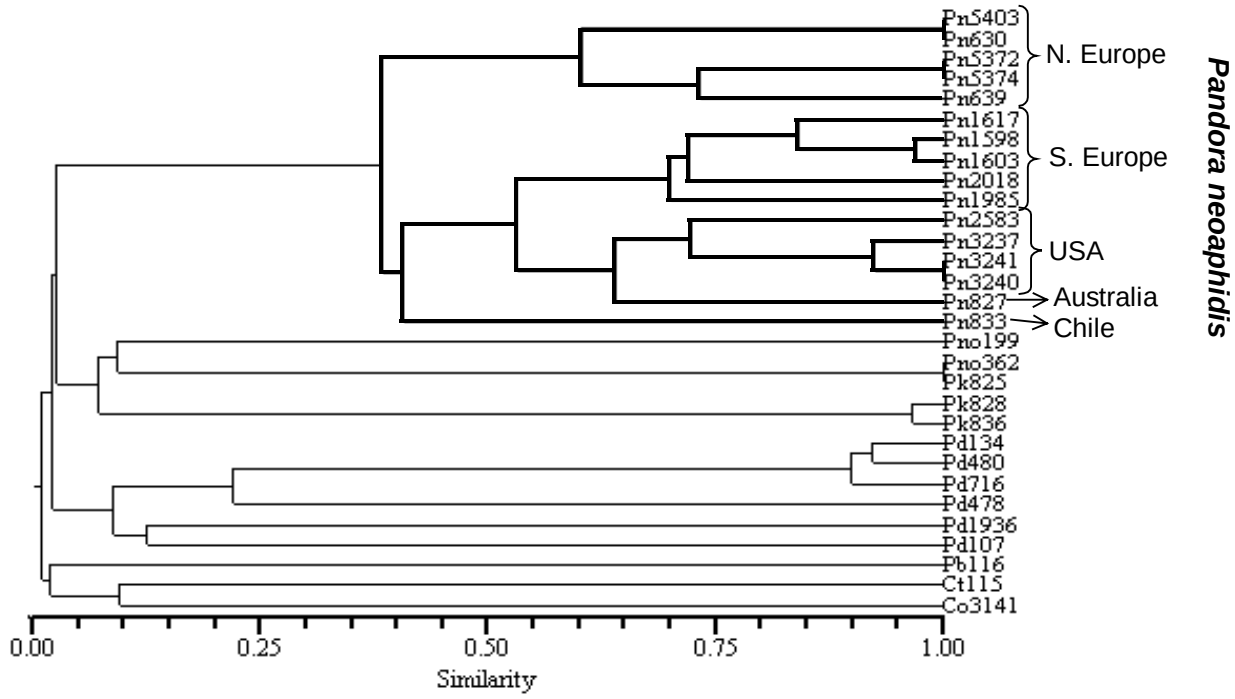

Figure 2.2
Dendrogram based on RAPD fragment pattern of Pandora neoaphidis *and related species. Pn* = Pandora neoaphidis; *Pno* = Pandora nouryi; *Pk* = Pandora kondoiensis*; Pd* = Pandora delphacis*; Pb* = Pandora bullata*; Ct* = Conidiobolus thromboides *and Co* = Conidiobolus obscurus*. Bold lines are isolates belonging to* P. neoaphidis.

Isolates representing different groups in the RAPD-PCR analysis were selected for measurement of length and width of the primary conidia from *in vitro* material. Results are shown in table 2.4 and figure 2.3. Conidia length, width and length / width ratios were subjected to analysis of variance by Tukey's multiple comparison procedure. Analysis of variance showed a significant effect of isolate for both length and width measurements and for ratio of length to width (length: $F_{12, 259} = 268.0$, $P<0.0001$; width: $F_{12, 259} = 145.5$, $P<0.0001$; L/W $F_{12, 259} = 77.3$, $P<0.0001$). Differences between *P. neoaphidis* isolates were small compared to other *Pandora* species. However conidia from the isolate originating from Chile were significantly longer and conidia from the Australian isolate of *P. neoaphidis* were significantly broader. These two isolates were also those which differed the most from the rest of the *P. neoaphidis* isolates in the analysis of RAPD-PCR data. However measurements fell in all cases within the species

descriptions. Analysis of the RAPD-PCR data and the conidia measurements showed that the two species *P. kondoiensis* and *P. nouryi* possess huge variation.

Table 2.4

Pandora *spp. primary conidia (*in vitro*) dimensions (µm) and length-width ratio. Means within the same column followed by different letter are significantly different by analysis of variance (Tukey's procedure, P<0.05)*

Species and collection site	Isolate no.	No. of conidia	Mean length and SE (µm)	Mean width and SE (µm)	Mean ratio
Pandora neoaphidis					
Chile	833	20	27.7 (0.3) d	16.1 (0.2) b	1.7 de
Australia	827	20	34.8 (0.7) a	18.2 (0.4) a	1.9 c
USA	3241	20	31.8 (0.5) bc	16.8 (0.7) b	1.9 c
USA	2583	20	33.6 (0.9) ab	15.4 (0.4) b	2.2 b
France	1598	20	33.2 (0.5) abc	16.4 (0.4) b	2.0 bc
France	1617	20	30.8 (0.7) c	15.2 (0.3) b	2.0 bc
Denmark	5372	20	30.8 (0.7) c	16.4 (0.3) b	1.9 dc
Pandora nouryi					
USA	199	20	16.6 (0.4) fg	10.9 (0.3) de	1.5 f
Australia	362	20	16.1 (0.3) fg	9.7 (0.2) ef	1.7 ef
Pandora kondoiensis					
Australia	825	20	15.0 (0.2) g	9.2 (0.2) f	1.6 ef
Australia	828	20	18.2 (0.3) f	11.9 (0.2) cd	1.5 f
Pandora delphasis					
USA	3107	20	23.1 (0.2) e	12.2 (0.2) c	1.9 dc

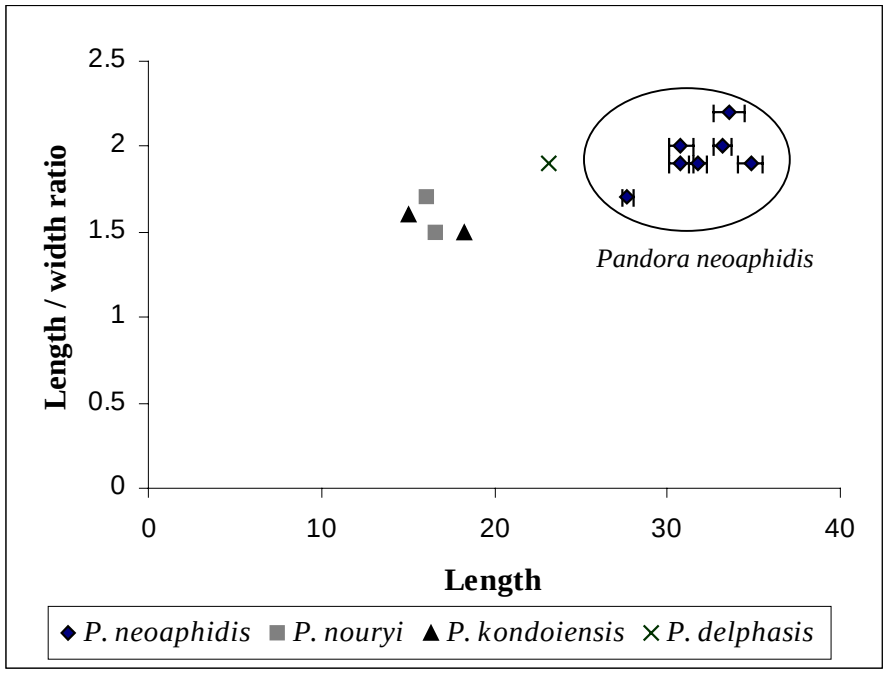

Figure 2.3

Length-width ratio as a function of length +/- S.E. (µm) for Pandora *spp. primary conidia* (in vitro).

2.4 **Conclusions**

Both morphological and DNA based methods have been used for characterisation of *P. neoaphidis* isolates, and our results proved that *P. neoaphidis* consists of several phenotypes and genotypes. Analysis of data showed that among the *P. neoaphidis* isolates RAPD grouping could be related to geographical origin whereas no clear conclusions could be drawn based solely on classical morphological measurements. No relationship between host insect and RAPD groups was observed.
In summary, we may conclude:

- The RAPD-PCR technique was implemented and further developed for the genus *Pandora*

- Both phenotypic and genotypic differences between isolates of *P. neoaphidis* were detected but not completely correlated

- For *P. neoaphidis* genotypic characters could be related to the geographical origin of the isolate (analysed from RAPD-PCR data)

- No relationship between host insect species and RAPD groups was observed

- The methods developed in this study are useful for environmental studies of insect pathogenic fungi, eg. monitoring of released strains of *P. neoaphidis*

3 Natural occurrence of entomophthoralean fungi infecting aphids

Epizootiology

Natural occurrence and dispersal of Entomophthorales is an important part of epizootiology (Tanada & Kaya, 1993). Emphasis thus far has been on the natural occurrence on pest insects. Only limited knowledge concerning the effects of other insects, soil, plants and air on dispersal and early development of epizootics of Entomophthorales is available. The aim of this chapter is to review the natural occurrence of Entomophthorales in different habitats such as aphids, soil and air. In chapter 4 this information will be used to discuss overwintering of the fungi, and chapter 7 will use this information to review the dispersal and development of epizootics of *P. neoaphidis*.

3.1 **Occurrence on aphids**

A number of publications document the natural occurrence of entomophthoralean fungi infecting aphids in Europe (Dean & Wilding, 1971; Veronina, 1971; Remaudière *et al.,* 1981; Keller & Suter, 1980, Feng *et al.* 1990, 1991; Bałazy, 1993; Sičev, 1992; Steenberg & Eilenberg; 1995), in USA (Feng *et al.* 1991; Steinkraus *et al.* 1995) and in Australia (Milner *et al.,* 1980). Altogether sixteen species of Entomophthorales have been described to infect aphids (table 3.1).

High prevalences

Worldwide, *P. neoaphidis*, *Entomophthora planchoniana* Cornu and in a few cases also *C. obscurus* are the most dominant pathogens on aphids feeding on annual crops with prevalences up to 80% (Dean & Wilding, 1971; Veronina, 1971; Remaudière *et al.,* 1981; Feng *et al.* 1990; Sičev, 1995; Steenberg & Eilenberg; 1995). In aphid populations feeding on perennial crops however, *Neozygites fresenii* (Nowakowski) Remaudière & Keller is often the dominating fungus species (Steinkraus *et al.* 1995; Nielsen *et al., in press*).

3.2 **Occurrence on cereal aphids**

In cereals

In Denmark aphids were surveyed during three years for the natural occurrence of Entomophthorales. Cereal aphids were sampled from both their primary and secondary hosts. Four entomophthoralean fungi were documented on cereal aphids when feeding on cereals: *P. neoaphidis*, *E. planchoniana, C. obscurus* and *Conidiobolus thromboides* Drechsler (table 3.2). Only *P. neoaphidis* and *E. planchoniana* caused epizootics. Prevalences of up to 50% were measured (Steenberg, Eilenberg & Nielsen, unpub.) This is in accordance with observations for cereal aphids in the United Kingdom, France and USA where these two fungi have also caused epizootics (Dean & Wilding, 1971; Remaudière *et al.,* 1981; Feng *et al.* 1990; Sičev, 1995; Steenberg & Eilenberg; 1995).

Table 3.1

Natural occurrence of Entomophthorales on aphids (Aphididae).

Family	Species	Host	Country	Reference
Ancylistaceae	*Conidiobolus coronatus*	*C* *O*	USA PL	Feng *et al.* (1990) Bałazy (1993)
	Conidiobolus obscurus	*M, S* *M, R, S,O* *M, R, S, O* *C* *M, R, O* *S, O* *M, R, S, O*	UK FR CH USA FR, USA, etc. PL DK	Dean & Wilding (1973) Remaudière *et al.* (1981) Keller (1987) Feng *et al.* (1990) Humber (1992) Bałazy (1993) Steenberg & Eilenberg (1995)
	Conidiobolus osmodes	*R, S, O* *O*	FR PL	Remaudière *et al.* (1981) Bałazy (1993)
	Conidiobolus thromboides	*C* *M, S, O* *O* *M, S*	USA USA, Rus, etc. PL DK	Feng *et al.* (1990) Humber (1992) Bałazy (1993) Steenberg & Eilenberg (1995)
Entomophthraceae	*Entomophaga pyriformis*	*O*	PL	Bałazy (1993)
	Entomophthora chromaphidis	*M, S* *o*	USA AU	Feng *et al.* (1990) Humber (1992)
	Entomophthora planchoniana	*S, M* *M, R, S,O* *M, R, S,O* *S, O* *R, S, O*	UK FR CH PL DK	Dean & Wilding (1973) Remaudière *et al.* (1981) Keller (1987) Bałazy (1993) Steenberg & Eilenberg (1995)
	Pandora kondoiensis	*O*	AU	Milner *et al.* (1983)
	Pandora neoaphidis	*S, M* *M, R, S, O* *M, R, S, O* *M, R, S, O* *M, S, O* *M, S, O* *M, R, S, O*	UK FR USA CH USA, EU, AU PL DK	Dean & Wilding (1973) Remaudière *et al.* (1981) Feng *et al.* (1990) Keller (1991) Humber (1992) Bałazy (1993) Steenberg & Eilenberg (1995)
	Pandora nouryi	*O* *O*	USA, AU PL	Humber (1992) Bałazy (1993)
	Zoophthora aphidis	*R, O* *O* *O*	CH CH PL	Keller (1991) Humber (1992) Bałazy (1993)
	Zoophthora occidentalis	*S* *O* *O*	USA USA PL	Feng *et al.* (1990) Humber (1992) Bałazy (1993)
	Zoophthora phalloides	*R, S* *R, O* *O* *O*	FR CH USA, FR, YU PL	Remaudière *et al.* (1981) Keller (1991) Humber (1992) Bałazy (1993)
	Zoophthora radicans	*M, S,* *M, O* *O*	USA USA, CH, FR PL	Feng *et al.* (1990) Humber (1992) Bałazy (1993)
Neozygitaceae	*Neozygites fresenii*	*O* *R, O* *O* *O*	USA CH PL DK	Feng *et al.* (1990) Keller (1991) Bałazy (1993) Steenberg & Eilenberg (1995)
	Neozygites lagniformis	*O*	USA, CL	Bałazy (1993)

	Neozygites microlofii	*O*	CH	Keller (1991)

C = Cereal aphids *M = Metopolophium dirhodum*
R = Rhopalosiphum padi *S = Sitobion avenae*
O = other aphid species than cereal aphids

On primary hosts

For aphids sampled on their primary hosts no infection could be documented during spring despite very intensive sampling from particularly bird cherry, the primary host for *R. padi* (table 3.2). However, an infection experiment showed that *R. padi* sampled from bird cherry in spring could easily be infected with *P. neoaphidis* under laboratory conditions (lethal time four to six days at 17°C). This means that *R. padi* feeding on bird cherry does not come into contact with inoculum in spring. In autumn all of the four species mentioned above were found on aphids sampled from primary hosts. Moreover *N. fresenii* and *Zoophthora phalloides* Batko were also found on *R. padi* (table 3.2) (Steenberg, Eilenberg & Nielsen, unpub.).

Table 3.2
Natural occurrence of Entomophthorales on cereal aphids feeding on different hosts in Denmark (Steenberg, Eilenberg & Nielsen, unpub.).

Season	**Host plant**	**Aphid species**	**No. of incubated aphids**	**No. of fungus-killed aphids**	***Pandora neoaphidis***	***Entomophthora planchoniana***	***Conidiobolus obscurus***	**Other *Conidiobolus* speices**	***Neozygites fresenii***	***Zoophthora phalloides***
Summer	Cereal crops	*S. avenae*	>10000	>1000	⊕	⊕	x	x		
Summer	Cereal crops	*R. padi*	>5000	>500	x	⊕	x			
Summer	Cereal crops	*M. dirhodum*	57	7	x		x	x		
Autumn	*Prunus padus*	*R. padi*	>1000	197	x	x	x	x	x	x
Autumn	*Hórdeum murinum*	*S. avenae*	~100	~20	x					
Spring	*Prunus padus*	*R. padi*	>10000	0						
Spring	*Hórdeum murinum*	*S. avenae*	50	0						

x = Fungus documented
⊕ = Fungus documented to cause epizootics

3.3 **Occurrence in soil**

Latteur (1977) documented the presence of active inoculum of *P. neoaphidis* and *C. obscurus* in soil immediately following an epizootic, while Corremans-Pelseneer *et al.* (1983) additionally documented *Condiobolus coronatus* (Costantin) Batko and *Conidiobolus* sp. Other than these studies, no information was found in the literature.

Occurrence in Denmark

In this study we investigated the occurrence of Entomophthorales infecting aphids by baiting soil samples with *S. avenae*. Infection of *S. avenae* by *P. neoaphidis*, *C. obscurus* and *Conidiobolus* sp. from soil was documented.

3.4 **Occurrence in air**

Hamilton (1959) was the first to estimate the aerial concentration of entomophthoralean fungi. It was found that the concentration of Entomophthorales conidia was highest between July and September. Diurnal periodicity was also measured and the maximum conidial number occurred between 5:00 a.m and 1:00 p.m. However the results were based on a small number of entomophthoralean conidia and were never determined at the species or even genus level. Wilding (1970) also measured the diurnal periodicity of the occurrence of Entomophthorales and found the largest concentration of Entomophthorales conidia in the air typically between 5:00 a.m. to 7:00 a.m. and furthermore that the concentration was correlated with humidity and sunrise. The smallest concentration occurred between 2:00 p.m. and 5:00 p.m., when the air was usually driest. Steinkraus *et al.* (1996) found generally found the same diurnal periodicity for primary conidia of *N. fresenii* in the air over a cotton field during epizootics in the cotton aphid *Aphis gossypii* Glover.

3.5 **Conclusions**

This chapter highligted current knowledge from our studies and from the literature on the natural occurrence of Entomophthorales infecting aphids with emphasis on cereal aphids in particular. With respect to basic observations of such occurrences, much information is available in the literature, however only limited detailed data are found.

Based on our studies in particular, we may conclude:

- The epizootic potential of Entomophthorales to infect aphids is high

- In Denmark the following entomophthoralean fungi were documented on cereal aphids when feeding on cereals: *P. neoaphidis*, *E. planchoniana*, *C. obscurus* and *C. thromboides* . For *R. padi* feeding on the winter host (*P. padus*) *N. fresenii* and *Z. phalloides* were also found, indicating different yearly life cycles for these two Entomophthorales compared to the species first mentioned

- The species *P. neoaphidis* and *E. planchoniana* both caused significant mortality in cereal aphid populations in Denmark. Prevalences of up to 50 % were measured

- The interactions between host plant, host aphid species, fungus species and time of the year are important for the development of epizootics

4 Survival of entomophthoralean fungi during winter

Cereal aphids are only present each year from May through October in Denmark. This means that Entomophthorales which attack cereal aphids must survive in the environment for at least six months out of every twelve.

4.1 **Survival structures**

Survival structures

The fungus may survive either as (1) resting spores (Burger & Swain, 1918; Batko, 1964ab; Remaudière & Hennebert, 1980; Remaudière & Keller, 1980; Keller, 1987,1991; Bałazy, 1993), (2) hyphal bodies (Keller, 1997a; Feng *et al.,* 1992), (3) conidia or 'loriconidia' (Weiser & Batko, 1966; Latteur & Randall, 1986) or (4) in anholocyclic aphid populations (Byford & Reeves, 1969; Wilding, 1973). The survival strategy depends on the fungus species (table 4.1).

Table 4.1
Suggested survival structures for aphid pathogenic Entomophthorales

Developmental stage	**Fungus**	**Reference**
Resting spores	*C. coronatus*	Bałazy, 1993
	C. obscurus	Remaudière & Keller, 1980
	C. osmodes	Bałazy, 1993
	C. thromboides	Bałazy, 1993
	E. pyriformis	Bałazy, 1993
	E. chromaphidis	Burger & Swain, 1918
	E. planchoniana	Keller, 1987
	P. nouryi	Remaudière & Hennebert, 1980
	Z. aphidis	Remaudière & Hennebert, 1980
	Z. occidentalis	Batko, 1964b
	Z. radicans	Batko, 1964a
	N. fresenii	Keller, 1991
	N. microlofii	Remaudière & Keller, 1980
Hyphal bodies	*E. planchoniana*	Keller (1997a)
	P. neoaphidis	Feng *et al.* (1992)
Conidia	*C. obscurus*	Latteur (1980)
	P. neoaphidis	Latteur & Randall (1986)

4.2 **Resting spores**

Resting spores

Among the aphid pathogenic fungi resting spores have been described as occurring *in vivo* for all species except *P. neoaphidis, P. kondoiensis, Z. phalloides* and *Neozygites lageniformis* (Thaxter) Remaudière & Keller (Thaxter, 1888; MacLeod & Müller-Kögler, 1973; Remaudière & Keller, 1980; Keller, 1987; Keller, 1991; Bałazy, 1993). Only limited information is available for the last species mentioned, and resting spores may occur.

4.2.1 **Development of resting spores**

A variety of factors are found to promote the development of resting spore, including abiotic parameters such as temperature (Shimazu, 1979; Milner & Lutton, 1983; Glare *et al.,* 1989; Hajek & Shimazu, 1996, Thomsen 1999), light (Thomsen, 1999), humidity (Glare, *et al.,* 1989) and biotic factors such as the fungal isolate (Glare *et al.*, 1989; Hajek, & Shimazu 1996; Thomsen, 1999), fungal density (Glare *et al.*, 1989; Hajek & Shimazu, 1989) host (Ben Ze'ev & Uziel, 1979), host age (Wilding & Lauckner, 1974; Shimazu, 1979; Steinkraus & Kramer, 1989; Hajek & Shimazu, 1989) and sex of host (Thomsen, 1999)

4.2.2 **Resting spore germination**

The timing of resting spore germination, which can take place over a longer period seems to be correlated with host, pathogen, temperature, humidity and light (table 4.2). Often a period of cold is required. Nonetheless the exact requirements for initiating germination have not been completely elucidated. Resting spores can remain infective for several years under field conditions.

Table 4.2
Entomophthoralean resting spores dormancy requirements (modified after Hajek, 1997).

Fungal species	**Conditions necessary for initiation of germination**	**Reference**
Conidiobolus obscurus	3 – 7 months at ≥ 95% RH	Perry & Latgé (1982)
Conidiobolus thromboides	No dormancy required	Soper *et al.* (1975)
Furia crustosa	2–4°C for 3 months in moist soil	Perry & Fleming (1989b)
Neozygites fresenii	5–14°C for 5–14 days at high RH	Ben-Ze'ev *et al.* (1990)
Pandora bullata	≤ 20°C for two months	Perry (1988)
Zoophthora radicans	4°C for 2 months in moist soil or at 100% RH	Perry & Fleming (1989a)
Zoophthora canadensis	> 12 hours light every 24 hours after 32 days at 4°C	Wallace *et al.* (1976)

4.3 **Hyphal bodies**

Hyphal bodies

Under cool and dry conditions it is possible for *P. neoaphidis* to survive for several months as hyphal bodies in cadavers without affecting the virulence of the conidia produced when the cadavers are moved to warmer and more humid conditions (Wilding, 1973; Courtois & Latteur, 1984; Latteur *et al*., 1985). Humidity and temperature in Denmark will probably never be consistently low enough during autumn and winter to ensure survival as hyphal bodies. However in cadavers of the pea aphid *A. pisum*, Feng *et al.* (1992) observed a special kind of hyphal bodies occurring late in the season. The hyphal bodies were spherical and clearly distinguishable from the regular hyphal bodies. While the appearance of these spherical hyphal bodies increased late in the season Feng *et al.* (1992) suggested that this kind of hyphal body may function as an overwintering form in the life cycle of *P. neoaphidis*. Therefore, Feng *et al* (1992) concluded that *P. neoaphidis* survives the winter months in the form of hyphal bodies on plant substrates rather than in the soil.

For *E. planchoniana* Keller (1997a) observed thick walled hyphal bodies in populations of *Drepanosiphum acerinum* (Walker). The proportion of infected aphids developing thick walled hyphal bodies instead of conidial infections was shown to increase during fall. Keller (1997a) also showed that in spring it was possible to infect healthy aphids with *E. planchoniana* from aphids containing thick walled hyphal bodies.

4.4 **Conidia and 'loriconidia'**

Conidia

Studies of conidial survival have primarily been concentrated on *P. neoaphidis* due to the lack of resting spores in this species. On fresh oilseed rape leaves Schofield *et al*. (1995) showed that conidia of *P. neoaphidis* remain infective of up to 32 days after incubation at 5°C and 85% r.h. and only up to 16 days after exposure to winter field conditions. It is therefore unlikely that conidia on leaf surfaces are the only overwintering mechanism. Nevertheless, conidia left on the surface of the soil are probably able to remain infective for a longer period. On the surface of soil under wet and dark conditions Latteur & Randall (1986) documented that primary conidia were able to produce replicate conidia for 24 days at 20°C and for 6 - 8 months at 5°C. They concluded that this must be the way that *P. neoaphidis* survives during winter. Morgan (1994) found in agreement with Latteur & Randall (1986) that primary conidia of *P. neoaphidis* were able to produce secondary conidia for 16 days at 18°C and for at least 8 months at 5°C on soil kept at a water holding capacity of 50%. However, Morgan (1994) also ran the experiment at 10°C and found that replicate conidia were only produced for one month. Morgan (1994) concluded that at least in Britain the winter temperature is not consistently low enough for the survival of *P. neoaphidis* as conidia. For *C. obscurus* it has been shown that conidia showered onto the surface of nonsterile soil can produce replicate conidia for several months and that these conidia can actually infect aphids (Latteur, 1980).

'Loriconidia'

Weiser & Batko (1966) observed in their studies of *Conidiobolus destruens* (Weiser & Batko) Ben-Ze'ev & Kenneth thick walled external conidia and descibed them as 'loriconidia'. They suggested that this structure was an alternative way of winter survival for Entomophthoralean fungi.

To investigate the survival structure of *P. neoaphidis* we incubated non-sporulating cadavers of *S. avenae* on sterilised soil in darkness at 5°C. After one month we examined the cadavers. In addition we examined conidia produced *in vitro* on solid media after approximately three months of storage. Around the stored cadavers we observed conidia similar to those Weiser & Batko (1966) earlier described as 'loriconidia' for *C. destruens*. From the stored *in vitro* cultures, thick walled conidia were observed as well. Thick walled hyphal bodies were not observed in this study.

4.5 **Anholocyclic aphid populations**

Anholocyclic aphid

Finally it has been suggested that *P. neoaphidis* survives in anholocyclic aphid populations via continuous conidial infections (Byford and Reeves, 1969; Wilding, 1973). Byford and Reeves (1969) found in spring that the peach-potato aphid, *Myzus persicae* Sulz. and the mangold aphid, *Rhopalosiphoninus staphylae* Koch, were infected with *P. neoaphidis* at

their overwintering place, beet clamps. They concluded that *P. neoaphidis* was able to survive on aphids in clamps prior to dispersal in spring. In anholocyclic aphid populations in northern littoral France *P. neoaphidis* has been observed throughout the year even during winter (Remaudière *et al.*, 1981). However winter temperatures are much higher there than in Denmark.

4.6 Soil as environment for survival of *Pandora neoaphidis*

Objective

The environment for survival, independant of the survival structure, may be either soil, leaves, trunks or in anholocyclic aphid populations. In this study we surveyed soil as a natural source of inoculum for Entomophthorales infecting aphids, particularly with reference to the winter survival of *P. neoaphidis.*

Methods

During 1997, 1998 and 1999 soils were sampled before immigration of aphids to their summer hosts. Soil from the surface was sampled underneath bird cherry in an organically grown beet field with winter wheat as the previous crop, and in permanent grass. Bioassays were conducted to evaluate infection of *S. avenae* by Entomophthoralean fungi in the soil samples. For each bioassay between 40 and 50 petri-dishes with soil samples were used for screening of fungi once or twice per week for four weeks. Between each bioassay soil samples were incubated at 17^{o}C and 12:12 L:D, until the next bioassay began. For each bioassay twelve 3rd-4th instar *S. avenae* nymphs were placed on the soil samples for 18 hours and then transferred to winter wheat seedlings and incubated at 20^{o}C. The aphids were monitored daily for one week to detect infection with entomophthoralean fungi (for more detailed information concerning materials and methods see Appendix C).

Entomophthoralean fungi present in soil

The percentage of soil samples (data from all localities are pooled) containing inoculum, as evidenced by *S. avenae* infection, is shown as a function of days after sampling in table 4.3. This study documented that *P. neoaphidis* and *C. obscurus* were present in soil from different ecosystems prior to immigration of cereal aphids in spring. Since the aphids were not always infected immediately following sampling of the soil, it is likely that the inoculum is dormant or quiescent during the winter. The breaking of dormancy or quiescence is thought to be a very complex process controlled by the fungus species as well as by temperature and humidity.

Table 4.3
Entomophthoralean fungi on soil surfaces as evidenced by Sitobion avenae *infection after 18 to 24 hours of contact with the soil. The percentages of soil samples which caused infection with entomophthoralean fungi are shown as a function of time. The soil samples were incubated at 17^{o}C and 12:12 L:D in 1997 and 14:10 L:D in 1998 and 1999.*

Days after sampling[1]	*Pandora neoaphidis*			*Conidiobolus obscurus*			*Conidiobolus* sp.		
	97	98	99	97	98	99	97	98	99
0	0.0	8.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0
2	0.0	-	0.0	0.0	-	4.0	0.0	0.0	0.0
7	4.5	4.0	22.9	0.0	16.0	4.2	0.0	0.0	2.0
14	6.8	0.0	11.6	8.0	16.0	14.0	0.0	0.0	0.0

[1] Days after sampling refer to the day when aphids were in contact with the soil.
- = Soil not tested.

4.7 **Conclusions**

The population interaction between aphids and fungi from Entomophthorales is complex. The fungi need winter survival structures and the fungi must furthermore adapt to the aphid population biology, which may include alternating summer and winter hosts. The initiation of infection during spring, however, is very important since it may be the determinant for the spring and early summer prevalence levels in cereal aphid populations.

In summary, we may conclude:

- Three different winter survival stages have been documented in Entomophthorales which infect aphids: resting spores, hyphal bodies and conidia

- The species *P. neoaphidis* has never been reported to produce resting spores, but seems to survive by means of hyphal bodies and/or conidia

- For some species within Entomophthorales, a dormancy period is needed before resting spores can germinate during spring

- The soil environment was shown to be a reservoir for winter survival structures of *P. neoaphidis* and *C. obscurus*

- Factors governing the initiation of infection in aphid populations in spring are still only partly understood

5 *In vivo* and *in vitro* isolation and growth of entomophthoralean fungi

5.1 Introduction

Information concerning factors important for *in vivo* and *in vitro* isolation and growth is important for both basic studies and the host-pathogen relationship as well as for the development of mass production methods.

5.2 *In vivo* isolation and growth

Isolation

For some of the entomophthoralean fungi the only means for growing the fungus is by using living host insects. The first step in running such an *in vivo* culture of any Entomophthorales involves identification and isolation. Usually one cadaver is placed over a glass slide under humid conditions to allow sporulation. The fungus is then identified while it is ensured that only one fungus species has invaded the insect tissue. Thereafter the cadaver can be used to start an *in vivo* culture. Preferably a culture is always initiated from a single cadaver to obtain an isolate which is as homogeneous as possible.

Growth

Methods for *in vivo* culturing of Entomophthorales are described for example in *Entomophthora schizophorae* Keller & Wilding in Keller infecting flies (Kramer & Steinkraus, 1981; Eilenberg, 1987) and *Neozygites floridana* (Weiser & Muma) Remaudière & Keller infecting mites (Smitley *et al.*, 1986). The principle is to permit conidia produced from sporulating cadavers to fall into a cage with healthy insects or onto plant material and then later add healthy insects. After some days, depending on temperature, host species and fungus, some of the insects exposed to condia die as a result of the fungus and begin to sporulate. A new cycle of disease can then be started from these insects.

5.2.1 *In vivo* culturing of *Pandora neoaphidis* infecting aphids

Pandora neoaphidis

The method for maintaining an *in vivo* culture of *P. neoaphidis* on *S. avenae* developed in this project is illustrated in figure 5.1.

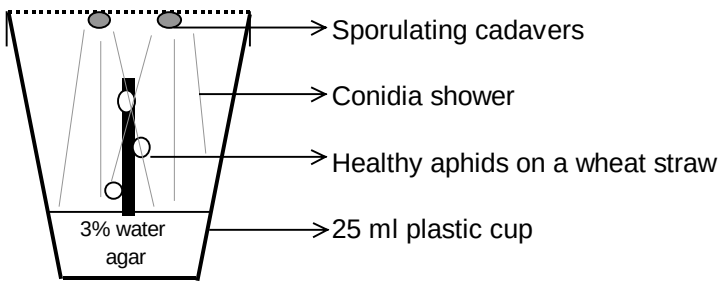

Figure 5.1
Set-up for in vivo *transmission of* Pandora neoaphidis *to healthy aphids.*

Between one and four sporulating cadavers of *S. avenae* were fixed with vaseline to the lid of a 25-ml plastic cup with 3% water agar in the bottom to keep humidity high. Six to ten healthy aphids were then transferred to a straw of winter wheat placed in the water agar. The cup was closed and incubated at 17-20^{o}C. From day four following inoculation the aphids in the cup were checked daily and infected aphids were removed and used to establish a new cycle of disease. One of the most important matters in this system is to maintain humidity close to 100% (Wilding, 1969).

5.3 *In vitro* isolation and growth

5.3.1 Isolation

Isolation

The first attempts to cultivate entomophthoralean fungi *in vitro* were done by discharging conidia from a cadaver into different kinds of media (Sawyer, 1929; Müller-Kögler, 1959; Gustafsson, 1965). Rockwood (1950) was the first to isolate *P. neoaphidis* by transferring sporulating hyphae to an egg medium as early as 1934. MacLeod (1956) developed a new method for isolating Entomophthorales where he surface sterilised non-sporulating cadavers and transferred the whole insect to the medium (figure 5.2). For small insects such as aphids this method is still recommended (Keller, 1994; Papierok & Hajek, 1997).

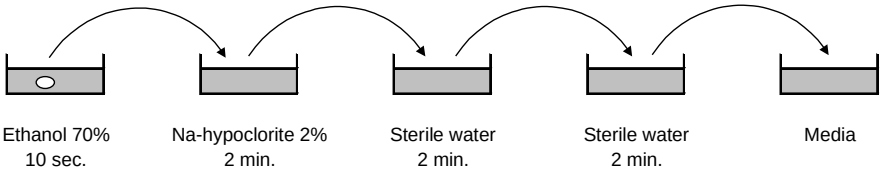

Figure 5.2
In vitro *isolation of Entomophthorales using the whole cadaver method (modified after Keller, 1994).*

Isolation from surface sterilised resting spores formed inside a cadaver has also been reported (Tyrell & MacLeod, 1975; Papierok & Hajek, 1997). Finally *in vitro* isolation has been carried out from the vegetative stages such as protoplasts or hyphal bodies by collecting small amounts of haemocoel with a syringe from an infected but still living insect (Papierok & Hajek, 1997).

For isolation both liquid and solid media have been used. Some species, eg. *E. muscae,* are best isolated in liquid media (Eilenberg *et al.*, 1992).

5.3.2 Nutritional requirements for *in vitro* growth

First attempts to grow Entomophthorales in vitro

Classical media, such as Sabouraud dextrose agar (SDA) or Sabouraud maltose agar (SMA), are not suitable for the majority of Entomophthorales. Sawyer (1929) and later Müller-Kögler (1959) both recommend coagulated egg yolk from hens as the best substrate for *in vitro* cultivation. Müller-Kögler (1959) also succeeded to in growing an unidentified species of Entomophthoraceae on milk agar, oat milk agar, beef extract, peptone yolk agar and potato pieces, however growth was never as significant as on the coagulated egg yolk media. Media based on coagulated egg yolk are still very common for isolation and growth of many species of Entomophthorales. Studies on the influence of the different lipid and protein fractions of egg yolk on the growth of several species of Entomophthorales proved that egg yolk does not contain any specific nutrients qualitatively required for Entomophthorales (Latgé & Bièvre, 1976; Latgé *et al.* 1978).

Later Latgé (1982) concluded that Entomophthorals primarily grow well on egg yolk because it is highly concentrated in nutrients with a water content of only 50% and furthermore because highly concentrated carbon is not linked to high osmotic pressure.

Carbon

Glucose has primarily been used as the carbon source, although maltose, fructose, thalose and glycerol have also been shown to be acceptable whereas sucrose is not (Latgé, 1975; Latgé *et al.*, 1978). In some cases sources other than glucose have been shown to be superior. This is for example the case for *Batkoa apiculata* (Thaxter) Humber and *Erynia curvispora* (Nowakowski) Remaudière & Hennebert (Gustafsson, 1965). Other carbon sources may be used in some cases. Latgé and Bièvre (1976) reported that *C. obscurus, C. thromboides* and others were able to use fatty acid as the carbon the source.

Nitrogen

Optimum growth is obtained on complex media of amino acids or protein hydrolisates as nitrogen sources. (Latgé, 1975). No species are able to utilise nitrate as the nitrogen source (Gustafsson, 1965; Latgé, 1982).

Vitamins and salts

Latgé & Sanglier (1985) showed for *C. obscurus* that Mg and to lesser extent Zn and Mn stimulated the formation of azygospores. They also showed that sulphur must be added in a reduced or oxidized form and phosphate must be present in the culture medium. Vitamins do not seem to have a significantl impact on growth (Dunphy & Nolan, 1982).

Serum

For some Entomophthorales, fetal serum has been shown to be essential, especially for those fungi which are growing as protoplasts. (Dunphy & Nolan, 1979; 1982).

Insect haemolymph

Grundschober *et al.* (1998) showed that insect haemolymph was mandatory for sustained growth of *Neozygites parvispora* (MacLeod & Carl) Remaudière & Keller, a pathogen to many thrips. This means that for some entomophthoralean fungi at least one putative growth factor is present in the haemolymph. The growth factor however has not yet been found.

The nutritional requirements for *in vitro* growth of insect pathogenic Entomophthorales are summarised in figure 5.3.

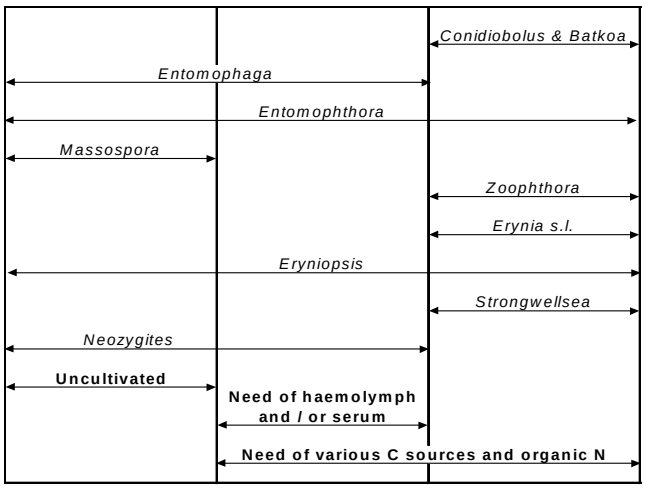

Figure 5.3
General table of nutritional requirements for in vitro *growth of Entomophthorales infecting insects (modified after Latgé, 1982).* Erynia sensu lato *includes species belonging to the genera* Erynia, Pandora *and* Furia.

5.3.3 **Physical requirements for *in vitro* growth**

Solid contra liquid media

Routine *in vitro* culturing depends on the developmental stage of the fungus required. Hyphal bodies grow both on solid and in liquid media while protoplasts only grow in liquid media (Papierok & Hajek, 1997). Entomophthoralean species growing only *in vitro* as protoplasts are necessarily kept in liquid media. Other species, which represent the majority, can be maintained on solid media.

Temperature

Gustafsson (1965) determined the temperature cardinal points for *Pandora dipterigena* (Thaxter) Humber, *P. nouryi*, *P. neoaphidis* and *Conidiobolus thromboides* on solid media and found that growth for all species was better at 21°C and 24°C than at 5 °C, 10 °C and 28°C. At 28°C the growth of all strains of *P. neoaphidis* was either very poor or absent. It is however remarkable that *P. neoahidis* grew even at 5°C. Robinson (1986) determined that the optimal temperature for colony radial growth of *P. neoaphidis* was 20°C.

pH

In general Entomophthorales are tolerant of pH levels between 6 and 7 with an optimum of approximately 6.5 for the species investigated so far. At pH below 5 and above 8 growth is remarkably depressed (Gustafsson, 1965; Latgé *et al*. 1977; Dunphy & Nolan, 1979; Latgé & Sanglier, 1985; Robinson, 1986). For *P. neoaphidis* the fastest growth rate has been obtained between pH 6.0 and 7.0 (Gustafsson, 1965; Robinson, 1986). The optimal pH corresponds well with the pH usually found in insect haemolymph. Gustafsson (1965) concluded that *P. neoaphidis* has a relatively narrowly limited pH optimum compared with species from the genus *Conidiobolus.*

5.3.4 **Vegetative growth of *Pandora neoaphidis* in liquid cultures**

Background

Earlier studies have shown that it is possible to cultivate *P. neoaphidis in vitro* on both solid media and submerged in liquid media for most of the media mentioned above. For commercial use of *P. neoaphidis,* liquid media are preferable due to faster and more homogeneous growth of the fungus compared to solid cultures. However, many problems are still to be resolved before the fungus can be used as a biopesticide. One of the most significant problems of growing *P. neoaphidis* in liquid media is the formation of pellets and / or heavy wall growth on the culture flasks (Robinson, 1986; Gray, 1990). The factors, which are supposed to influence the uneven growth are both mechanical and physiological. Gray (1990) suggested that an increasing oxygen concentration dissolved in the media decreases the degree of pellet formation. Formation of pellets can also be prevented by disruption of the inoculum in the flasks (Gray, 1990). This may be done either by using baffles in the culture flasks or by increasing the agitation speed. This may simultaneously increase the amount of dissolved oxygen in the media. Finally the source/morphology of the inoculum may play a significant role.

Objectives

The primary objective of this investigation was to elucidate the effects of mechanical stress by using baffles and different agitation speeds (60, 120, 180, 240 rpm). Furthermore, the effects of inoculating liquid media with different sources of inoculum (conidia, hyphal fragments from a liquid culture or homogenised pellets from a solid culture) were also investigated.

Methods

The effects were measured as weight of biomass after different times of growth, glucose concentrations in the filtrate mentioned above, measurements of length of hyphal bodies, ability to sporulate and finally a visual determination of wall growth and pellet formation. One isolate of

P. neoaphidis (KVL 98-11) was chosen for these experiments. The isolate was isolated from *R. padi* in Copenhagen, Denmark, in the summer of 1998 and maintained *in vitro* since. In all experiments liquid cultures were grown in YEMG containing 1.6% (w/v) glucose (BioChemica), 1.0% (w/v) yeast extract (Oxoid) and 10% (v/v) pasteurised semi-skimmed milk (Coop). All cultures were grown at 20^{o}C in constant darkness.

Results

To elucidate the effects of the source of inoculum liquid media was inoculated with conidia, hyphal fragments from a liquid culture or homogenised pellets from a solid culture. Best results with respect to fast and homogeneuos growth were obtained when the liquid culture was inoculated from another liquid culture. When inoculating with conidia, growth started very slowly and it took more than a week before growth was detectable. When inoculating with homogenised pellets from a solid culture, many fungal cells were destroyed indicating that much more inoculum must be used in order to obtain good and fast growth. Furthermore, growth was not more homogeneuos than when using liquid culture for inoculation.

The effect of baffles was a greater speed of growth at all four agitation speeds (Figure 5.4), however very heavy wall growth occurred particularly at the highest agitation speeds. After 180 hours of growth at 240 rpm the wall growth reached the lid of the flasks. This suggests that other types of baffles should also be tested.

For flasks without baffles speed of growth was very slow at 60 and 120 rpm, whereas the speed of growth was acceptable at both 180 and 240 rpm.

As shown in table 5.1 agitation speed also had a significant effect on sporulation when measured as number of conidia per mm^2 over 24 hours.

During the 144 hours that the experiment took place no remarkable changes in the distribution of length of hyphal fragments were observed (figure 5.5).

Table 5.1
Effects of agitation speed and baffles on sporulation of Pandora neoaphidis *over 24 hours (isolate KVL 98-11) in YEMG in 20 ml flask culture (100 ml Erlenmeyer flask, 20^{o}C; constant darkness.). wb = with baffles; ob = without baffles.*

Treatment	Wb 60	Wb 120	Wb 180	Wb 240	Ob 60	Ob 120	Ob 180	Ob 240
No. of conidia per mm^2	700	252	1273	552	49	864	910	1191
No. of conidia per dry weight	152	35	260	139	26	200	132	179

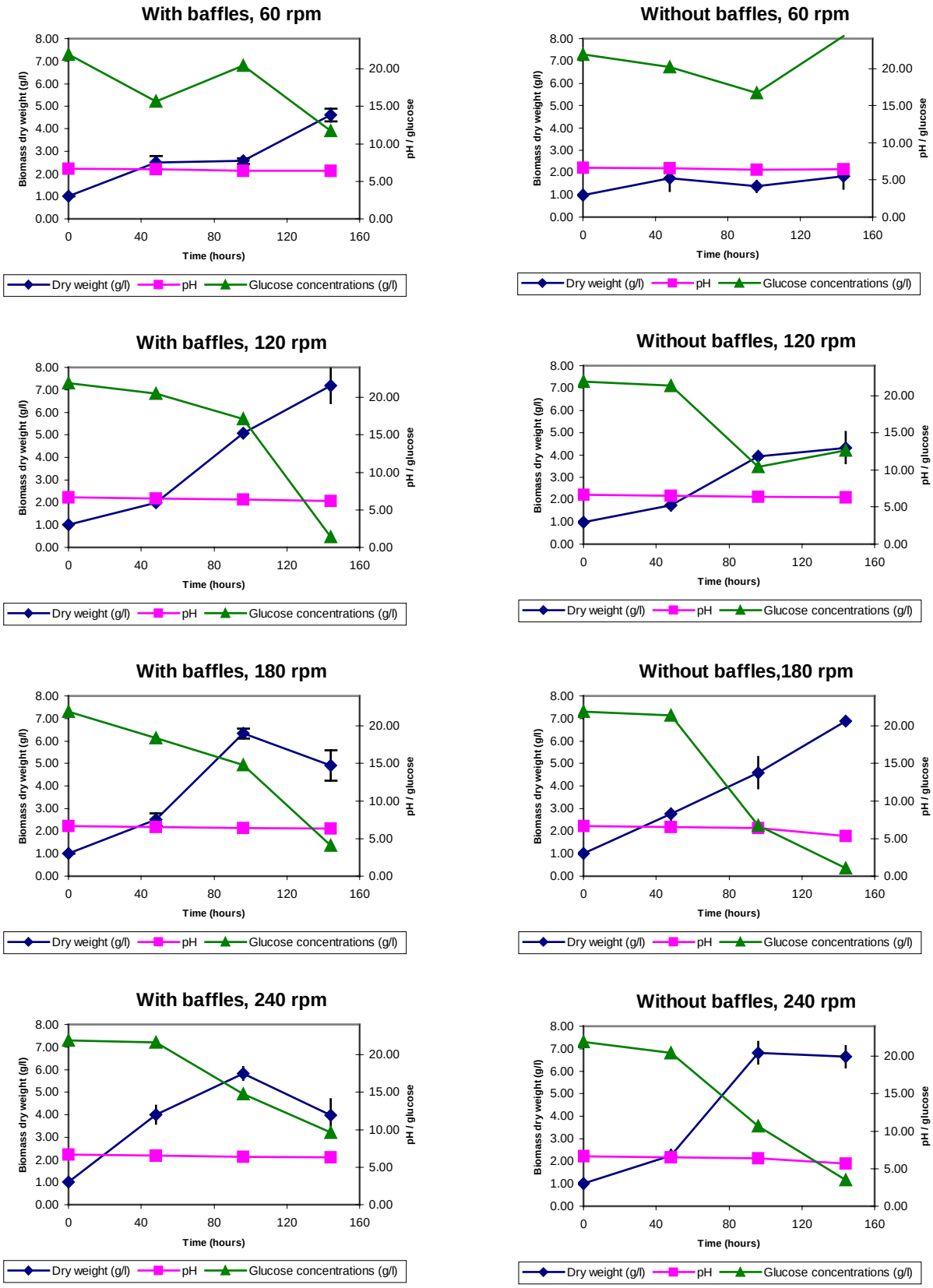

Figure 5.4
Effects of agitation speed and baffles on growth of Pandora neoaphidis *(isolate KVL 98-11) in YEMG in 20 ml shake flask cultures (100 ml Erlenmeyer flasks; 20°C; constant darkness).*

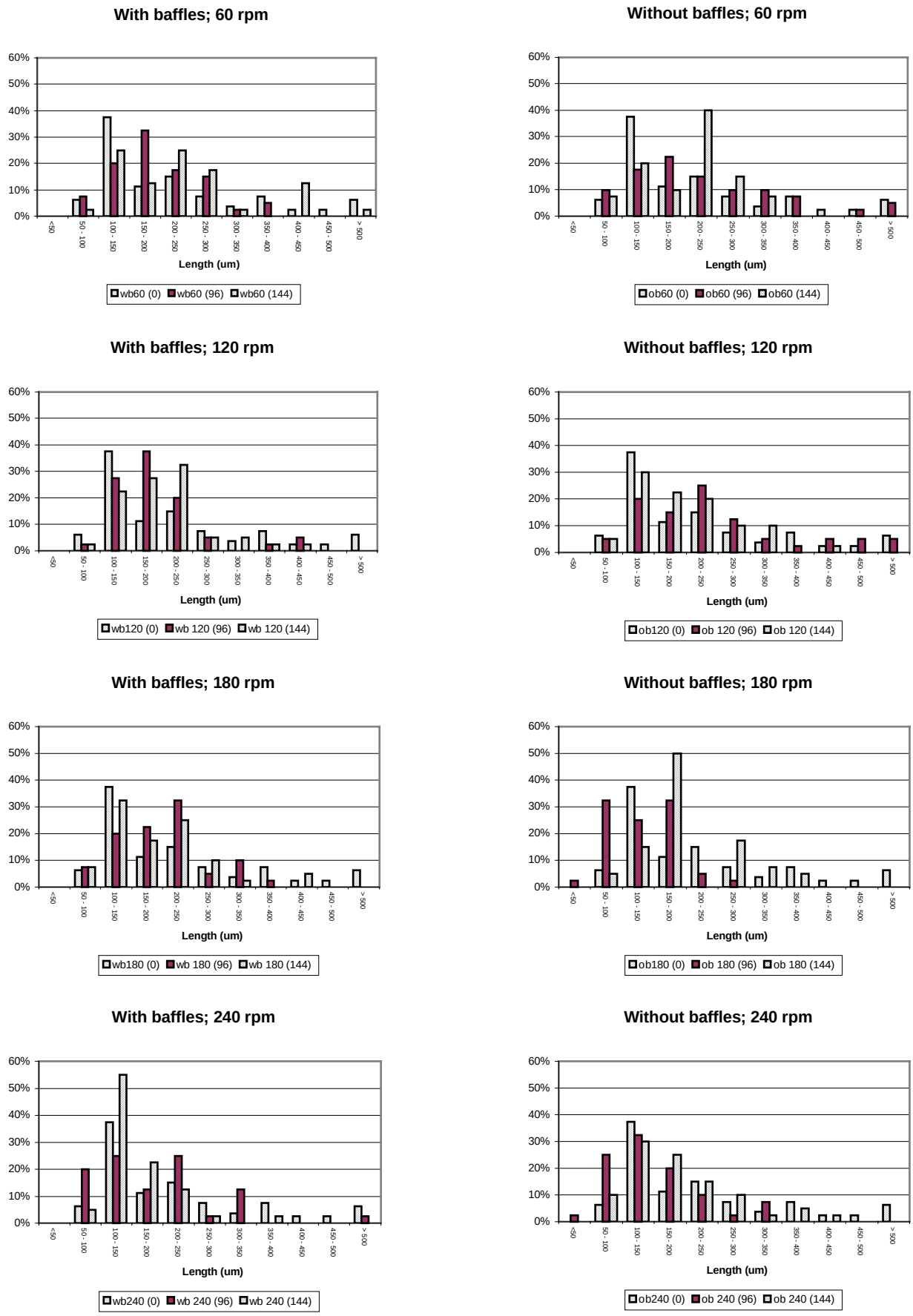

Figure 5.5
Effect of agitation speed and baffles on growth of Pandora neoaphidis *(isolate KVL 98-11) in YEMG in 20 ml flask cultures (100 ml Erlenmeyer flasks; 20°C; constant darkness).*

5.4 **Formulation and application**

The use of *P. neoaphidis* for augmentative biological control by dispersing sporulating cadavers or infected aphids gave mixed results (Wilding, 1981; Wilding *et al.,* 1990). Another and probably less laborious method is to use *in vitro* produced material. Latteur & Godefroid (1983) and later Sylvie *et al.* (1990) conducted a number of experiments where unformulated hyphal bodies were sprayed to control aphids. However, in none of the cases was adequate control obtained.

Very recently a Swiss group of insect pathologists began working on formulating Entomophthorales, primarily *P. neoaphidis.* They found that encapsulating hyphal bodies into sodium alginate beads gave promissing results concerning conidiation and infectivity against aphids in the laboratory (Shah *et al.* 1998). Glasshouse experiments carried out by the same Swiss group have shown promising results concerning biological control of the potato aphid (*Macrosiphum euphorbiae* (Thomas) with alginate formulated *P. neoaphidis* (Tuor *et al.,* 1999).

5.5 **Conclusions**

In summary, we may conclude:

- Fungi from Entomophthorales can be kept in culture *in vivo,* and many species have also been isolated *in vitro*

- A method for *in vivo* cultivation and growth in *S. avenae* was developed for *P. neoaphidis* and was used for *in vivo* transmission studies

- Fungi from Entomophthorales are diverse in their requirements to *in vitro* isolation and growth and some require additions such as serum and/or insect haemolymph

- *P. neoaphidis* was isolated *in vitro* from Danish aphids and furthermore grown in simple, liquid media in the laboratory.

- Experimental work on *in vitro* growth of *P. neoaphidis* demonstrated that baffles supported growth but also resulted in fungus growth on the wall of the flasks

- Agitation speeds between 180 and 240 rpm were successful in promoting the growth of *P. neoaphidis*

- In Switzerland, experiments with *P. neoaphidis* have led to improved formulations and successful, though still preliminary, biological control of aphids in glasshouses

6 Virulence of *Pandora neoaphidis* against *Sitobion avenae* and *Rhopalosiphum padi*

6.1 **Bioassay methodology**

Pathogenicity and virulence

Pathogenicity of an insect pathogen is defined as the ability to produce disease in insects (Lacey, 1997). The proof of pathogenicity is the first step towards studies on virulence. The virulence of an insect pathogen is defined as the quality or property of being virulent (Lacey, 1997). Assessment of the virulence of an insect pathogen requires quantitative studies on dose-response relationships.

LC_{50} and LD_{50}

In studies of dose-response relationships, the terms LC_{50} and LD_{50} are the most common expressions of virulence. LC_{50} is the concentration of a given insect pathogen required to kill 50 % of the test insect population within a given period of time, whereas LD_{50} expresses the dose required to kill 50% of the population. With respect to fungi from Entomophthorales, LC_{50} is the appropriate term since the methodology only allows an estimate of the concentration used and not the dose actually received by the test insects (Papierok & Hajek, 1997).

LT_{50}

The term LT_{50} is defined as the time period required to kill 50 % of the test insect population when subjected to a given concentration or dose of an insect pathogen. The term is often used a quantitative expression of the virulence of fungi from Entomophthorales. The shorter the lethal time documented for a fungus against the test insect, the higher the virulence. Furthermore, the term LT_{50} provides much of the information needed to understand the dispersal of the disease in the insect population and the dynamics of the host-pathogen system. In several cases, both LC_{50} and LT_{50} were measured for a host-pathogen system using a fungus from Entomophthorales as the pathogen and aphids as the test insect (Papierok & Hajek, 1997).

Bioassay methodology

Bioassays with Entomophthorales can be performed in different ways. The most common method is the 'conidia shower' method (Papierok & Hajek, 1997, Eilenberg, 1999). The test insects are subjected to a conidial shower (sporulating cadavers or conidia from *in vitro* cultures). The concentration of conidia is expressed as number of conidia per mm^2 or similar. Using insect cadavers, less precise expressions such as number of cadavers are sometimes used to express the concentration of conidia. Conidial showers from cadavers or *in vitro* cultures do in any case reflect the natural route of infection, although studies using Entomophthorales may suffer from limited replicability.

Injections into the insect haemolymph of protoplasts or hyphal bodies have also been used to assess virulence (Papierok & Hajek, 1997). This method gives high replicability but does not reflect the natural route of infection.

6.2 **Bioassays against *Sitobion avenae* and *Rhopalosiphum padi***

LC_{50} of P. neoaphidis *against* S. avenae

Our experimental determination of the LC_{50} of *P. neoaphidis* against *S. avenae* was carried out using an *in vitro* isolate of *P. neoaphidis* and the conidia shower method. Cohorts of ten aphids from two clones (green or brown alates or apterous) were subjected to a conidial shower. We varied the time under conidial shower (0, 5, 10, 15, 25, 35, 50, 80, 120, 150 and 180 minutes) to obtain different concentrations of conidia, and the number of conidia per mm^2 was calculated. Incubation took place at 18°C and mortality was recorded after seven days.

Data are shown in table 6.1. There was no significant difference between the two clones. For both clones the LC_{50} values for alates were significantly lower than for apterae.

Table 6.1
Susceptibility of morphs and clones of Sitobion avenae *to infection of* Pandora neoaphidis *(Isolate KVL-634), expressed by LC_{50} values*

Morph and clone	**Slope**	**S.E.**	**LC_{50}-value** (conidia/mm^2)	**96% C.L.** (conidia/mm^2)
Green apterae (HI 95)	0.36	0.08	2.88	2.19-3.79
Green alates (HI 95)	0.43	0.05	0.85	0.60-1.20
	0.32	0.06	1.77	1.35-2.32
Brown apterae (HF92A)	0.36	0.04	2.62	2.27-3.01
	0.32	0.04	3.56	2.99-4.25
Brown alates (HF92A)	0.58	0.14	1.47	1.09-1.98

LT_{50} of P. neoaphidis *against* S. avenae

The experiments to assess LT_{50} were performed using a similar methods as in the LC_{50} studies. All test insects were however subjected to conidia for 60 minutes before being placed in different temperatures. Data from the experiments using green alates are shown in figure 6.1.

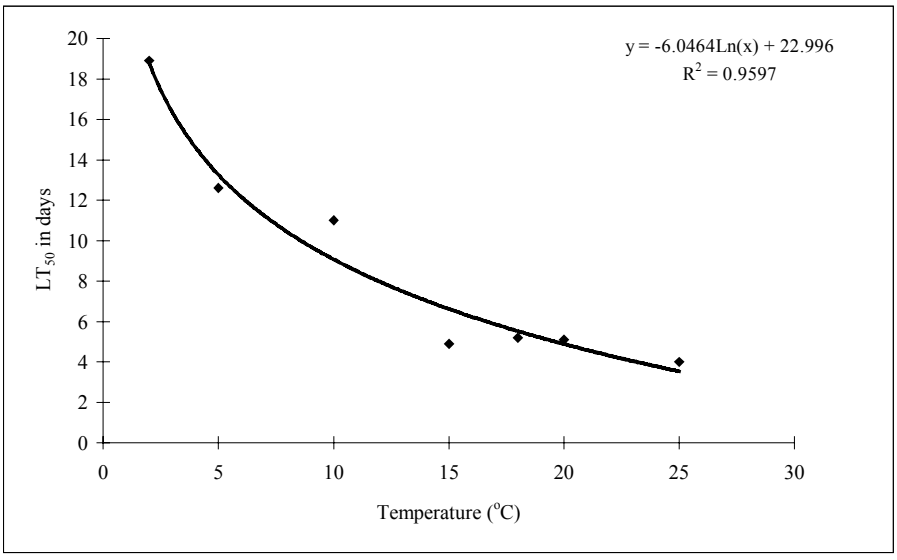

Figure 6.1
Lethal time (LT_{50}) of alates of the green Sitobion avenae *clone (H1) infected with* in vivo *material of* Pandora neoaphidis *(Isolate KVL-634).*

As seen in figure 6.1, lethal time is highly dependent on temperature. At 25°C, the lethal time is approximately 4 days, while incubation at 5°C results in a lethal time of 12-14 days.

A comparison of the lethal time at 18°C for *S. avenae* subjected to *P. neoaphidis* is seen in table 6.2. The calculated lethal times varied between 6.6 and 7.5 days between the morphs and clones, though the differences were not significant.

Table 6.2
Lethal time at 18°C of morphs and clones of Sitobion avenae *infected with* in vivo *material of* Pandora neoaphidis *(Isolate KVL-634).*

Morph and clone	**LT_{50}-values (days)**	**S.E.**
Green apterae (HI 95)	7.2	0.3
Green alates (HI 95)	6.8	0.2
Brown apterae (HF92A)	6.6	0.2
	7.5	0.7
Brown alates (HF92A)	6.8	0.2

Virulence of P. neoaphidis *against* R. padi

Similar experiments were carried out using *R. padi* as the receptor. These data are included in table 6.3.

Table 6.3
LT_{50} data with aphids and Entomophthorales from our experiments and from the literature.

LT_{50} data on aphids/ Entomophthorales

Fungus	**Temp. (°C)**	**Lethal time (days)**	**Host**	**Reference**
P. neoaphidis	2	18.9	*Sitobion avenae*	*This study*
	5	12.6	*Sitobion avenae*	*This study*
	8	13.8	*Acyrthosiphon kondoi*	Milner & Bourne, 1983
	10	11.0	*Sitobion avenae*	*This study*
	10	9.1	*Acyrthosiphon kondoi*	Milner & Bourne, 1983
	10	12.0	*Sitobion avenae*	Schmitz *et al.*, 1993
	12	7.8	*Acyrthosiphon kondoi*	Milner & Bourne, 1983
	15	4.9	*Sitobion avenae*	*This study*
	15	5.1	*Acyrthosiphon kondoi*	Milner & Bourne, 1983
	15	8.9	*Sitobion avenae*	Schmitz *et al.*, 1993
	16	5.4	*Brevicoryne brassicae*	Sivčev, 1994
	18	5.2	*Sitobion avenae*	*This study*
	18	4-5	*Rhopalosiphum padi*	*This study*
	20	5.1	*Sitobion avenae*	*This study*
	20	4.4	*Sitobion avenae*	*This study*
	20	3.5	*Acyrthosiphon kondoi*	Milner & Bourne, 1983
	20	4.1	*Sitobion avenae*	Schmitz *et al.*, 1993
	20	3.6	*Acyrthosiphon pisum*	Brobyn & Wilding, 1977
	20	3-4	*Acyrthosiphon pisum*	Wilding, 1969
	21.5	3.4	*Brevicoryne brassicae*	Sivčev, 1994
	25	4.0	*Sitobion avenae*	*This study*
	25	5.2	*Sitobion avenae*	Schmitz *et al.*, 1993
	30	-	*Sitobion avenae*	*This study*
	35	-	*Sitobion avenae*	*This study*
E. planchoniana	20	5.0	*Aphis fabae*	Brobyn & Wilding, 1977
C. obscurus	18	3-4	*Rhopalosiphum padi*	*This study*
	18	3-4	*Sitobion avenae*	*This study*
	20	3.1	*Acyrthosiphon pisum*	Brobyn & Wilding, 1977
	20	2-3	*Acyrthosiphon pisum*	Wilding, 1969

Table 6.3 shows our data on LT_{50} along with the literature data for *P. neoaphidis* or other fungi from Entomophthorales and different aphid species. The three fungus species are the most common species on cereal aphids. As can be seen in the table, the generel tendency is that at incubation temperatures above 20°C the lethal time is 3-5 days, whereas it is much higher at lower temperatures. Our data on *S. avenae* give precise information on this relationship and also contributes to knowledge concerning the general understanding of aphid/fungus relationships.

6.3 **Conclusions**

Discussíon and conclusions

The bioassay methodologies used for Entomophthorales were modified in order to obtain precise data on *S. avenae* and *R. padi.* The method allowed a comparison between different morphs and clones for the aphids, and the data obtained were satisfactory with respect to reproducibility.

A comparison of our data with the literature data (table 6.3) demonstrated that aphids are quickly killed by fungi from Entomophthorales, but there are differences in the measured LT_{50} data depending on aphid species, aphid morph, fungus species or even isolate and incubation temperature.

In summary, we may conclude:

- It was possible to implement and further develop a technique for dose-response studies on cereal aphids. The method can be used in other studies on aphids and Entomophthorales
- Differences in responses between morphs of *S. avenae* to infection of *P. neoaphidis* were detected
- The lethal time for *S. avenae* subjected to *P. neoaphidis* varied strongly with temperature
- Data on *S. avenae* and *R. padi* were comparable to the literature data from the same aphid species and other pest aphid species
- Fungi from Entomophthorales are highly virulent to cereal aphids

7 Dynamics of *Pandora neoaphidis* epizootics in *Sitobion avenae* populations

7.1 Definitions of some model terms

Models

An understanding of the dynamics of fungal diseases of insects is critical to the development of epizootiological theories as well as potential prediction of infection levels for pest management purposes (Hajek *et al.*, 1993). A typical integrated pest management (IPM) system model consist of three parts: (1) a biological-conceptual model developed from the literature, (2) a mathematical representation of that framework, and (3) a computer program implementing the mathematics (Brown, 1987).

However, the dynamics of fungal epizootics are poorly understood, and factors necessary for the development of epizootics have only been identified for very few host-pathogen systems (Hajek, & St. Leger, 1994). As far as we know, only one IPM model takes entomopathogenic fungi into account, namely a system consisting of the cotton aphid (*A. gossypii*) and *N. fresenii* in Arkansas, USA (Steinkraus, 1998) which is a system quite different from the cereal system in Denmark.

7.2 A biological conceptual model for the dynamics of *Pandora neoaphidis* in *Sitobion avenae* populations

A biological conceptual model

The dynamics of *P. neoaphidis* in populations of *S. avenae* are illustrated using a flow diagram in figure 7.1. In the figure both the aphids and the fungus are divided into three 'stage variables' each according to the principle phases of the disease. These stage variables are connected with several 'development rates'. The development rates are dependent on abiotic factors such as temperature, moisture and solar radiation ('forcing variables'). Moreover the forcing variables may determine whether an infection can occur (Benz, 1987; Carruthers & Hural, 1990). Since most of the processes are occurring in a cereal field, the crop development as well as agricultural practices must be included as forcing variables in the model. Some pesticides have shown to have a negative impact on the development of epizootics (Zimmerman, 1978; Wilding & Brobyn, 1980). As discussed in chapter 4, soil is important as a reservoir of *P. neoaphidis* and thus for early development of epizootics with *P. neoaphidis.*

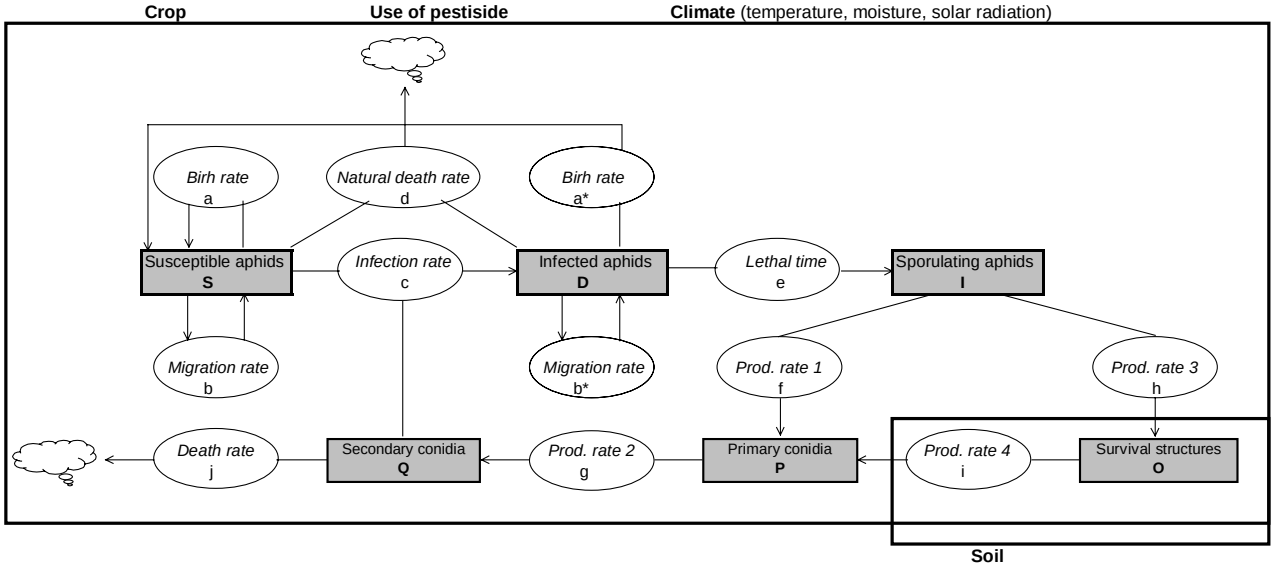

Figure 7.1
Flow diagram of an epizootiological model of the dynamics of Pandora neoaphidis *in a population* of Sitobion avenae*. Grey rectangles are stage variables, ovals are developmental rates and the two large rectangles are forcing variables.*

7.2.1 **Stage variable**

Aphids

Aphids are divided into the following categories: susceptible healthy aphids (S), infected aphids (D) and sporulating cadavers which spread the infective conidia (I). The sporulating cadavers only exist long enough to release the infectious units and will then disperse from the system. In other models the insect population has been divided into as many as five categories according to the development of the disease within the insect (eg. Schmitz *et al.*, 1993; Ardisson *et al.*, 1997). However, the categories described here are those which can usually be estimated from field data.

In this model it is assumed that all host stages and morphs are equally susceptible to aphid pathogens. This is however a simplification since results analysed in this project demonstrated a difference in prevalence between alate and apterous *S. avenae* within cereal fields (Appendix D). Bioassays were conducted and it was shown that this difference in prevalence was due to differences in susceptibility rather than differences in behaviour. To keep the model as simple as possible, however this is not taken into account and may not be important for the dynamics since most alate aphid probably emigrate before they die from the disease.

Fungus

The pathogen is divided into survival structures (O), primary conidia (P) and secondary conidia (Q). The cadavers produce either conidia, or survival structures or both. The conidia are disharged actively from cadavers and cause secondary infections among the aphids. Whether the primary and / or the secondary conidium is the infective unit is still not known. However, for *E. muscae* it has been proven that secondary conidia are about 200 times as infective as primary conidia (Bellini *et al.,* 1992) and the same can be true in our system. As mentioned in chapter 4 the question of how *P. neoaphidis* survive is poorly understood. The fungus must however, overwinter in some stage and initiate primary infections in spring.

7.2.2 **Effect of abiotic factors on development rates**

Abiotic factors do not act independently but as a complex of processes. Therefore, analysis of single environmental factors on the epizootics of fungal diseases is not always successful. Moreover, true quantitative values are difficult to obtain in nature since the microenvironment of an insect and/or a pathogen may differ considerably from the average measurable conditions of the environment (Benz, 1987). The effects of abiotic factors on the dynamics of disease development are discussed in greater detail below.

Birth rate (a)

In spring *S. avenae* migrate to cereal fields when accumulated day degrees reach 1150-1250D^{o} (above a threshold of 0^{o}C) and they begin to reproduce (Hansen, 1995). The reproduction rate is primarily temperature dependent. Because only horisontal transmission of fungus diseases occurs, both susceptible and infected hosts produce susceptible nymphs which moult through a normal sequence of nymph instars and finally become adults. The fecundity decreases for an infected aphid compared to a healthy one. The closer the aphid gets to death the larger the decline in fecundity (Schmitz *et al.*, 1993).

Migration rate (b)

It is assumed that the migration rate for infected aphids follows the migration rate for healthy aphids since information in the litterature concerning change in migration behaviour is found.

Infection rate (c)

Before infection of an aphid can occur, contact is necessary between the infective unit and the aphid. Tanada & Kaya (1993) reported that the spread of disease depends on both densities of host and infective unit. An increase in one or both pools enhances the probability of contact between spores and aphids. In contrast, Wilding (1975) found that aphid density had only minor importance in the transmission of infection. Missionnier *et al*. (1970) similarly concluded that epizootics were independent of host density. An analysis of Danish data has shown that density of aphids only has an impact when the density is low and that a threshold value probably exists. In addition to contact between spores and aphids, suitable temperatures and relative humidities are necessary for germination of spores and for penetration of the aphid integument (Benz, 1987).

Conidia of *P. neoaphidis* require a humid environment to be able to germinate and penetrate the integument. At high temperatures, a shorter time with free water is required to ensure optimal germination and penetration (Milner & Bourne, 1983). Germination occurs at any temperature between 10 ^{o}C and 20^{o}C (Milner & Bourne, 1983), and outside of this interval no results have been found in the literature.

Lethal time (e)

Our experiment showed that the lethal time increased as temperature decreased for aphids infected with *P. neoaphidis.* The relationship between lethal time and temperature can be expressed by the equation:

$$LT_{50} = -6.05*\ln(\text{temp.}) + 23.0$$

$$R^2 = 0.96$$

Production rate 1 (f)

Dromph *et al.* (1998) demonstrated a positive correlation between dry weight of *S. avenae* cadavers and total production of *P. neoaphidis* primary conidia at 18^{o}C. The relationship can be expressed as:

$$\text{Conidia produced} = 3186*\text{dry weight (in mg)} + 11412$$

$$R^2 = 0.97$$

Climatic factors, particularly humidity and temperature act on conidia discharge (Benz, 1987). Dromph *et al.* (1998) demonstrated that temperature had a significant impact on sporulation, particularly on total production, which increased with temperature. At 18°C most conidia were produced within the first twelve hours following death of the aphid. Wilding (1969) proved that *P. neoaphidis* only sporulated when RH was at least 90 %. Within this range, the numbers of spores increased with increasing humidity. Light apparently does not affect the sporulation of *P. neoaphidis* (Milner, 1981).

Production rate 2 (g)

It is assumed that climatic factors, particularly humidity and temperature act on the capacity of secondary conidia production in the same way as in primary conidia.

Production rate 3 (h)

No quantitative data have been collected concerning the initiation of the survival structure. Temperature and nutritional conditions may play an important role (Latgé & Papierok, 1988) and probably the day length too.

Production rate 4 (i)

For some entomophthoralean fungi a dormancy period is required prior to germination (Latgé & Papierok, 1988). Our data suggest that quiescence or dormancy for the survival structure of *P. neoaphidis* is broken a long time before the aphids arrive to the field. Infection can thus start as soon as the aphids arrive.

Conidia death rate (j)

P. neoaphidis loses the infectivity at a rate dependent on humidity and temperature (Wilding, 1973; Brobyn *et al.*, 1987). Inoculum on leaves near the base of the plants remained infective longer than on leaves near the top (Broby *et al.*, 1985) probably as a result of less solar radiation.

7.3 **Conclusions**

In summary, we may conclude

- Models are essential for understanding the development of epizootics
- A biological conceptual model for *S. avenae* and *P. neoaphidis* was developed
- Data from our studies and literature entered into the model
- The model suggested that *P. neoaphidis* has significant potential for biological control of *S. avenae*, but that many factors can also be critical for success
- The concept for epizootic models can be used for other insect host - fungus relationships

8 General discussion and conclusions

8.1 Background

The background for this work was the need to develop alternative methods for pest insect control. Due to a general wish from the society to minimise the use of chemical pesticides, biological control may offer a sound, environmentally friendly alternative.

Choice of system

The cereal aphid/fungus system was chosen as a model system based on: 1) The importance of cereals in Denmark, 2) The difficulties in controlling aphids in cereals without chemical pesticides, and 3) Knowledge of the natural occurrence of entomopathogenic fungi obtained in an earlier project.

Aphids will in the future still be significant pest insects, and the desire to minimise the use of chemical pesticides has certainly not diminished during the project period (eg. the "Bichel Report" to the Ministry of Environment 1999).

The project proceeded in three ways: 1) New scientific information was obtained during the project period based on the specific experimental work, 2) Relevant literature was reviewed, and 3) A high level of information exchange was maintained with other research groups (in Denmark and internationally) during the period. The report contains both specific new data from our studies, a compilation of data from the literature, and the newest available data from other research groups, including the most recent findings reported at conference in August 1999.

8.2 General Perspectives

The national dimension

For the Danish cereal system, the set of data obtained represents a solid basis for future work directly addressing biological control. Two types of biological control are potentially possible: 1) A direct release of Entomophthorales as biopesticides and 2) An enhancement of the natural control of aphids with Entomophthorales. Both strategies were given attention in our studies. With respect to 1), the fungus *P. neoaphidis* has the highest potential at present. We were able to characterise, isolate and grow this fungus *in vivo* and *in vitro*, perform bioassays and develop parts of a model for this species in relation to cereal aphids.

However, before the fungus can be used directly as a biopesticide, a range of further experiments must involve: more efficient growth *in vitro*, formulation, release in the field, and evaluation of field release. Our studies have shown that the fungus should be subjected to further investigation in order to develop outdoor biological control.

Our attention on the winter survival of Entomophthorales provided us with significant information for a deeper understanding of the plant-aphid-fungus system. Based on the results we do not hesitate to propose that future use of Entomophthorales to control aphids should not solely be based on the biopesticide approach, but that strategy 2) should certainly also be seriously considered to enhance natural control (eg. earlier initiation of epizootics). Additional experimental work should in such cases include more in depth studies on the importance of cropping systems, surrounding hedges and other landscape structures for the development of epizootics.

The European dimension

During the project period, attention on Entomophthorales as biocontrol agents of aphids has increased significantly. In particular, the group at ETH, Switzerland, and the group at Rothamsted Exp. Station, UK, should be mentioned. We maintained close contact with these groups and performed experimental work in co-operation (e.g. growth *in vitro*). The increased attention on Entomophthorales has led to a European network cooperation from year 2000 (COST Action 842). Results from the Danish studies on cereal aphids contributed to the success of the application.

In the coming years, we plan to use the European network to ensure that our results will be used by the scientific society to assist in well-designed experiments for both the cereal aphid-Entomopthhorales system and for other insect-fungus systems as well.

In summary, conclusions from the project as a whole are as follows

- Morphological, pathobiological and molecular characterisation methods were developed for *P. neoaphidis,* and proved that these sets of methods are now operational for ecological studies and can be transferred to other systems (chapter 2)

- The natural occurrence of Entomophthorales in different ecosystems relevant to cereal aphids was described, and proved that a number of species from Entomophthorales occur on cereal aphids with *P. neoaphidis* and *E. planchoniana* being the most important species (chapter 3)

- The winter survival structures of Entomophthoralean fungi infecting cereal aphids were investigated. It was shown that the soil is an important reservoir for infective units during winter, and that the number of fungal species occurring on cereal aphids on their winter host plants is higher than in the crop (chapter 4)

- Methods for *in vivo* and *in vitro* isolation and growth of *P. neoaphidis* were implemented and further developed. It was shown that in host transmission studies, *in vivo* cultures are sufficient although effective growth *in vitro* can also be obtained (chapter 5)

- The virulence of *P. neoaphidis* against *R. padi* and *S. avenae* was investigated and it was shown that the different morphs varied with susceptibility and that the lethal time was strongly dependent on temperature (chapter 6)

- The interaction between *P. neoaphidis* and *S. avenae* was described by a biological conceptual model. The model confirmed that *P. neoaphidis* has potential for biological control of *S. avenae* (chapter 7)

- The set of data obtained are of special relevance for the cereal aphid - Entomophthorales system, however the methods used (eg. characterisation methods) may prove useful in other studies on insect host - fungal pathogen systems, e.g. ecological studies on the performance of a fungus following release in the field

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Appendix A

Morphological features of entomophthoralean fungi infecting aphid

Morphological features of entomophthoralean fungi infecting aphids.

Species	Primary conidia			Conidiophores	Sec. conidia	R. spores	Rhizoids	Cystidia	References
	Shape	Size	Nuclei						
Conidiobolus coronatus (Constantin) Batko	Globose	L:14.5-53 µm W:17-48.5 µm	>50	Unbranched	Like primary or microconidia	Present	Absent	Absent	Keller, 1987; Bałazy, 1993
Conidiobolus obscurus (Hall et Dunn) Remaud. & Keller	Spherical	L:33.5-44 µm W:28-36 µm	>50	Unbranched	Like primary	Present	Absent	Absent	Keller, 1987
Conidiobolus osmodes Drechsler	Globose to ovoid	L:25-37 µm W:22-30 µm	>50	Unbranched	Like primary	Present	Absent	Absent	Bałazy, 1993
Conidiobolus thromboides Drechsler	Globose	L:24-32 µm W:17.5-26.5 µm	>50	Unbranched	Like primary	Present	Absent	Absent	Bałazy, 1993
Entomophaga pyriformis (Thoizon) Bałazy	Pyriform	L:15-31 µm W:12-25 µm	20-30	Unbranched	Like primary	Present	Absent	Absent	Bałazy, 1993
Entomophthora chromaphidis Burger & Swain	Bell shaped	L:11-14 µm W:10-11 µm	4-6	Unbranched	Like primary	Present	Present	Absent	Burger & Swain, 1918; Humber & Feng, 1991
Entomophthora planchoniana Cornu	Bell shaped	L:15.5-19.5 µm W:12.5-16 µm	6-8	Unbranched	Like primary	Present	Present	Absent	Keller, 1987
Pandora kondoiensis (Milner in Milner, Mahon & Brown) Humber	Ovoid	L:16-24 µm W:10-12 µm	1	Branched	Like primary	Absent	Present	Present	Milner *et al*, 1983
Pandora neoaphidis (Remaud. & Henn.) Humber	Ovoid to Elliptic	L:21-32 µm W:11-14 µm	1	Branched	Like primary	Absent	Present	Present	Remaudière. & Hennebert, 1980
Pandora nouryi (Remaud. & Henn.) Humber	Ovoid	L:15-16.6 µm W:8-10.5 µm	1	Branched	Like primary	Present	Present	Present	Remaudière. & Hennebert, 1980
Zoophthora aphidis (Hoffmann in Fresenius) Batko	Subcylindrical to fusiform	L:28-32 µm W:9-12 µm	1	Branched	Like primary or capilliconidia	Present	Present	Present	Remaudière. & Hennebert, 1980
Zoophthora occidentalis (Thaxter) Batko	Spindle or almost cylindrical	L:30-35 µm W:7-8.5 µm	1	Branched	Like primary or capilliconidia	Present	Present	Pseudo-cystidia	Bałazy, 1993
Zoophthora phalloides Batko	Long cylindrical	L:26-34 µm W:6-8.5 µm	1	Branched	Like primary or capilliconidia	Present	Present	Pseudo-cystidia	Remaudière & Hennebert, 1980
Zoophthora radicans (Brefeld) Batko	Cylindrical	L:15-22 µm W:5.5-7.5 µm	1	Branched	Like primary or capilliconidia	Present	Present	Pseudo-cystidia	Remaudière & Hennebert, 1980
Neozygites fresenii (Nowakowski) Remaud. & Keller	Spherical	L:18.2-21.5 µm W:14.5-17.8 µm	4	Unbranched	Like primary or capilliconidia	Present	Absent	Absent	Keller, 1991
Neozygites microlophii Keller	Spherical to pyriform	L:24.1-25.5 µm W:17.6-18.8 µm	(4-) 5	Unbranched	Like primary or capilliconidia	Present	Absent	Absent	Keller, 1991

Appendix B

Random Amplified Polymorphic DNA- PCR

Isolation of DNA
Before isolation of DNA all fungi were grown for one to three weeks in Grace's insect culture media added 5% Fetal Bovine Serum (Gibco BRL) at 20^{o}C in constant dark. The mycelium was harvest after centrifugation under sterile conditions and DNA was isolated following the protocol of Hodge *et al.* (1995) but modified by a RNase treatment. After precipitation of nucleic acid with isopropanol RNA was degraded by resuspending the nucleic acid in 250 µl sterile TE buffer (10 mM Tris-HCL; 0.1 nM EDTA). 1:125 RNase PLUS was added (5 Prime → 3 prime, Inc.) and samples were incubated for 2 hours at 37^{o}C. Samples were then centrifuged at 13000 rpm at 5^{o}C for 20 minutes and ammonium acetate was added to a concentration of 5.0 M. DNA was precipitated overnight at –20^{o}C with 2.5 vol cold 95% ethanol, centrifuged at 13000 rpm and washed twice with 70% cold ethanol. DNA was resuspended in filter sterilised distillated water and quantified by UV spectrophotometry.

RAPD
Amplification reactions were performed in 1.5 ml centrifuge tubes with a total volume of 30 µl. Each reaction contained 1x supplied PCR Buffer (10mM Tris-HCl, 10mM KCl, Gibco BRL), 3 mM $MgCl_2$, 200 µM each of dATP, dCTP,dGTP and dTTP (Boeringer Mannheim), 0.5 µM primer, 5 ng genomic DNA and 3 units *Taq* polymerase (Gibco BRL). Reactions were overlaid with 30µl mineral oil and amplifications were performed in a MJ Research PTC-100 thermocycler with the following cycle parameters: an initial denaturisation of 2 min at 93^{o}C; 40 cycles of 1 min at 93^{o}C, 1 min at 36^{o}C; 2 min at 72^{o}C; a final extension of 7 min at 72^{o}C. Amplification products were separated by electrophoresis in 1.4% agarose gels with 1x TBE buffer at 44 v for 18 hours and detected by staining with ethidium bromide. For each tested primer two replicates were carried out.

Lengths of DNA fragments were measured and scored from photographs of the gels. Differences in intensity were not taken into account and products not appearing in both replicates were disregarded. Data was recorded as a binary matrix and phenetic similarity was calculated (UPGMA using Jaccard's coefficient) by use of the statistical software NTSYSpc (V.2.01e).

Appendix C

SOIL - A NATURAL SOURCE OF ENTOMOPHTHORALEAN FUNGI INFECTING APHIDS

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SOIL - A NATURAL SOURCE OF ENTOMOPHTHORALEAN FUNGI INFECTING APHIDS

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SUMMARY:

The aim of the project was to investigate whether soil is a natural source of inoculum of Entomophthoralean fungi infecting aphids, particularly with reference to winter survival of *Pandora neoaphidis* (= *Erynia neoaphidis*). Soil was sampled during spring 1997 from different ecosystems including organically grown fields and underneath birdcherry, the winter host for the birdcherry aphid *Rhopalosiphum padi*. It was documented that inoculum of *P. neoaphidis, Conidiobolus obscurus, Conidiobolus* sp. and *Basidiobolus* sp. was present in the soil, since grain aphids (*Sitobion avenae*) got infected with these species after 18 hours of contact to the soil. Additional laboratory studies indicate so far that the winter survival structure for *P. neoaphidis* may be thick walled conidia.

INTRODUCTION:

In Denmark the most frequent fungi infecting cereal aphids are *Pandora neoaphidis* (= *Erynia neoaphidis*), *Entomophthora planchoniana* and *Conidiobolus obscurus.* It is generally accepted that resting spores are the winter survival stage for *C. obscurus* and *E. planchoniana.* But resting spores have never been documented *in vivo* for *P. neoaphidis*. However, the fungus must survive somehow during winter since *P. neoaphidis* in most years is observed in cereal aphids soon after immigration of aphids into cereal crops (Dean & Wilding, 1973; Feng *et al.*, 1991; Steenberg & Eilenberg, 1995).

The fungus has been suggested as surviving the winter by conidial infection in anholocyclic aphid populations and then is dispersed to fields in early spring. So far *P. neoaphidis* infections have been observed in overwintering populations of the peach-potato aphid *Myzus persicae* and the mangold aphid *Rhopalosiphonius staphylae* (Byford and Reeves 1969, Wilding, 1973). Another

hypothesis is that the fungus overwinters in aphid cadavers as some specialized hyphal bodies. Such structures have been observed in populations of the pea aphid *Acyrthosiphum pisum* infected with *P. neoaphidis* (Feng *et al.*, 1992). Finally it has been suggested that *P. neoaphidis* may survive as conidia in either soil, on leaves or trunks of trees etc. (Latteur & Randall, 1989; Morgan, 1994). Soil has been shown to be a natural source of inoculum of *P. neoaphidis* and *C. obscurus* in fall (Latteur, 1977).

The aim of this work was to study soil as a natural source of inoculum for entomophthoralean fungi infecting aphids, especially with reference to the winter survival of *P. neoaphidis.*

MATERIAL AND METHODS:

During 1997 soils were sampled before immigration of aphids to their summer hosts on May 14 and June 3 from four different localities in Denmark: (1) a sparsely vegetated plot underneath birdcherry (*Prunus padus*), (2) a well vegetated plot underneath bird-cherry, (3) an organically grown beet field with winter wheat as the previous crop, and (4) a plot with permanent grass. On each sampling date respectively 40 and 48 soil samples (1-2 cm deep) were collected with a spoon without disturbing the soil, transferred to 3 cm petri dishes and then brought to the laboratory. Bioassays were conducted to evaluate infection of *S. avenae* by Entomophthoralean fungi at the soil samples. For each bioassay all 40 or 48 petridishes with soil samples were used for screening of fungi once or twice per week for four weeks. Between each bioassay soil samples were incubated at 17°C and 12:12 L:D, until the next bioassay began. For each bioassay twelve 3rd-4th instar *S. avenae* nymphs were placed on the soil samples for 18 hours and then transferred to winter wheat seedlings and incubated at 20°C. The aphids were monitored daily for one week to detect infection with entomophthoralean fungi.

On May 14 and June 3, 1997 more than 100 birdcherry oat aphids were sampled from the winter host *P. padus.* The aphids were incubated at 20°C and checked daily for one week for infection with entomophthoralean fungi.

To investigate the surviving structure of *P. neoaphidis* in greater detail, non-sporulating cadavers of *S. avenae* were incubated on soil in darkness at 5°C. After one month the cadavers were examined using a light microscope. Conidia produced *in vitro* on EYSMA were examined after approximately three months of storage.

RESULTS AND DISCUSSION:

Infection of *S. avenae* by *P. neoaphidis*, *C. obscurus*, *Conidiobolus* sp. and *Basidiobolus* sp. from soil were documented. The percentage of soil samples (data from all localities are pooled) containing active inoculum, documented by infecting aphids, are shown as a function of days after sampling (Table 1). Days after sampling refer to the day where aphids were in contact with the soil. Until day 7 after sampling no inoculum of entomophthoralean fungi was documented. The first infection to occur was *P. neoaphidis*. Day 7 after sampling *P. neoaphidis* infection was observed in approximately 5% of the soil samples. Day 14 after sampling it was documented that soils contained active inoculum of both *P. neoaphidis* and *C. obscurus.* Day 20 after sampling, the soil again contained active inoculum of both *P. neoaphidis* and *C. obscurus.* Inoculum of a *Basidiobolus* sp. was documented 26 days after sampling of the soil. Fungi from the genus *Basidiobolus* have never been described as aphid pathogens. However since the aphids were filled with hyphal bodies and the aphids were monitored daily it is assumed that *Basidiobolus* sp. caused the death of the aphids. Finally the soils contained active inoculum of a *Conidiobolus* species 29 days after sampling of the soil, probably *C. coronatus*. None of the *R. padi* sampled from birdcherry were infected with entomophthoralean fungi.

Table 1: Entomophthoralean fungi on soil surfaces documented by infection of *Sitobion avenae* after 18 hours of contact with the soil. Percent soil samples which caused infection with entomophthoralean fungi are shown as a function of time, the soil samples was incubated at 17°C and 12:12 L:D.

Days after sampling[1]	*Pandora neoaphidis*		*Conidiobolus obscurus*		*Conidiobolus* sp.		*Basidiobolus* sp.	
	May 14[2]	June 3[3]	May 14	June 3	May 14	June 3	May 14	June 3
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	5.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0
14	10.0	4.2	7.5	8.3	0.0	0.0	0.0	0.0
20	2.5	-	2.5	-	0.0	-	0.0	-
26	-	0.0	-	0.0	-	2.1	-	2.1
29	0.0	-	0.0	-	5.0	-	0.0	-

[1] Days after sampling refer to the day where aphids were in contact with the soil.

[2] Soil samples collected on May 14, 1997; 40 soil samples.

[3] Soil samples collected on June 3, 1997; 48 soil samples.

- = Soil not tested.

From the microscopy studies external thick-walled conidia were observed on the cadavers. The conidia were morphologically similar to what Weiser & Batko (1966) earlier described as 'loriconidia' for *Conidiobolus destruens*. From the stored *in vitro* cultures, thick walled conidia were also observed. Further investigation will show if these thick walled structures may serve to ensure winter survival for *P. neoaphidis.*

This study documented that *P. neoaphidis* and *C. obscurus* are present in soil from different ecosystems before immigration of cereal aphids in spring. Results from this survey suggest that *P. neoaphidis* survives the winter months as 'loriconidia' or in some other form than resting spores. Since the aphids were not infected right after sampling of the soils, it is likely that the inoculum is dormant or quiescent during the winter. The breaking of dormancy or quiescence is thought to be a very complex system controlled by the fungus species as well as by temperature and humidity.

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Appendix D

Poster presented at Society for Invertebrate Pathology 29th Annual Meeting, September 5, 1996. Cordoba, Spain.

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ODDS-RATIO, A USEFUL ESTIMATE IN INSECT EPIZOOTIOLOGY

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INTRODUCTION

An insect population often consists of several subpopulations (e.g. alate/apterous; female/male). The infection level of a particular disease measured as prevalence may differ in such subpopulations. To estimate the influence of insect pathogenic fungi as mortality factors it is thus important to make unbiassed sampling plans and take the potential differences into account. The Odds-ratio estimate is useful for expressing differences in prevalence in two subpopulations. The Odds-ratio estimate expresses the increase of risk (if any) of a particular disease in two subpopulations. For each population the Odds-ratio between the two subpopulations is the ratio between the Odds. An Odds-ratio of 1 means an equal infection level in two subpopulations (Mantel & Haenszel, 1959; Breslow & Day, 1980). The Odds-ratio estimate (ψ) is defined as

$$\psi = \frac{a/b}{c/d} = \frac{a \times d}{b \times c}$$

where

a	=	number of diseased individuals in subpopulation 1.
b	=	number of healthy individuals in subpopulation 1.
c	=	number of diseased individuals in subpopulation 2.
d	=	number of healthy individuals in subpopulation 2.

Data from a single sampling are often too sparse to allow conclusions concerning differences in prevalence between two subpopulations. Under the hypothesis that the Odds-ratio remains constant from strata to strata (e.g. sampling dates) it is possible to calculate a weighed common Odds-ratio estimate (ψ^*), from which conclusions can be drawn based on sound statistics.

There are several ways to calculate weighed common Odds-ratio estimates. The two most frequently are the Mantel-Haenszel estimate (ψ^*_{MH}) and the logit estimate (ψ^*_L) given as

$$\psi^*_{MH} = \frac{\Sigma\, a_i d_i / N_i}{\Sigma\, b_i c_i / N_i} \qquad \ln(\psi^*_L) = \frac{\Sigma w_i \ln \frac{a_i d_i}{b_i c_i}}{\Sigma w_i}$$

where
i = i^{th} strata (e.g. sampling date)
N_i = $(a_i + b_i + c_i + d_i)$
w_i = $(1/a_i + 1/b_i + c_i + d_i)^{-1}$

Both the Mantel-Haenzel estimate (ψ^*_{MH}) and the logit estimate (ψ^*_L) have some advantages and disadvantages but in most cases the values calculated using the two methods do not differ considerably.

It is possible to calculate confidence intervals for both the logit (ψ^*_L) and the Mantel-Haenzel estimate (ψ^*_{MH}) and by a Chi-square to test the null hypothesis that $\psi^* = 1$. It is, however also necessary to test the homogeneity of the Odds- ratio. This is done by a Chi-square test but with the null hypothesis that ψ^* = the calculated weighed common Odds-ratio.

We have used the analysis in two different host-pathogen systems. **(1)** To analyse differences in prevalence related to morph of the English grain aphid (*Sitobion avenae* F.) infected with *Erynia neoaphidis* Remaud. & Henn. **(2)** To analyse differences in prevalence related to sampling area of adult carrot flies (*Psila rosae* F.) infected with *Entomophthora schizophorae* Kell. & Wild.

EXAMPLE 1: *SITOBION AVENAE* INFECTED WITH *ERYNIA NEOAPHIDIS*

Aphids of different morphs (alate / apterous) were collected during three seasons by cutting tillers from Danish winter wheat fields. Aphids were incubated in the laboratory at 20°C in constant light. Mortality was recorded daily for seven days and dead aphids were removed and placed over glass slides under humid condition for conidia production and diagnosis.

For two of the seasons (1993 and 1995) the English grain aphid was the most abundant aphid species accounting for more than 90% of aphids present in the crop. The predominant insect pathogenic fungus was *E. neoaphidis*. During the epizootic period it was observed that the alates apparently were more infected than the apterous (e.g. 48% versus 36% for July 29, 1993).

Table 1 shows the estimates of the weighed common Odds-ratio (ψ^*) for differences in prevalence related to morph of the English grain aphid infected with *E. neoaphidis*. Both the Mantel-Haenzel estimate and the logit estimate are given together with their respective 95%-confidence limits. From this it appears that the values calculated with the two methods are nearly identical. Both estimates documented that alates were significantly (.05) more infected than apterous. The hypothesis of a constant Odds-ratio throughout the seasons was accepted.

Table 1: Analysis of the persistence in differences in prevalence of *Erynia neoaphidis* among different morphs of *Sitobion avenae* sampled in winter wheat (Denmark).

	Chi-square	ψ^*_{MH}	Confidence limit (95%) around ψ^*_{MH}	ψ^*_L	Confidence limit (95%) around ψ^*_L	Homo-geneity test
Sitobion avenae						
(Adult: alate/apterous)						
1993	0.5^{NS}	1.49	0.66-3.34	1.52	0.68-3.44	0.6^{NS}
1995	15.4**	3.01	1.72-5.24	2.66	1.53-4.64	7.2^{NS}
1993 & 1995	14.6*	2.46	1.56-3.90	2.23	1.41-3.53	6.0^{NS}

The logit estimate of the weighed common Odds-ratio (ψ^*_L) between alates and apterous was 2.23 based on eight sampling dates (strata) distributed over two seasons. There can be two different explanations for the differences in prevalence between alate or apterous namely behaviour or susceptibility. So far we suggest that the main reason must be differences in susceptibility since bioassay experiments have documented differences in susceptibility between different morphs of aphids (Dromph, *pers. comm.*). However, whether behaviour also has an influence has not yet been investigated.

One advantage of the weighed Odds-ratio estimate (ψ^*) is that data from different strata can be used and that 95%-confidence intervals thus are minimized. In figure 1 are shown the Odds-ratio estimates and the respective confidence intervals for alate/apterous of the English grain aphid. It is clear that a narrow 95%-confidence interval is obtained for the weighed common Odds-ratio compared to the intervals from a single date.

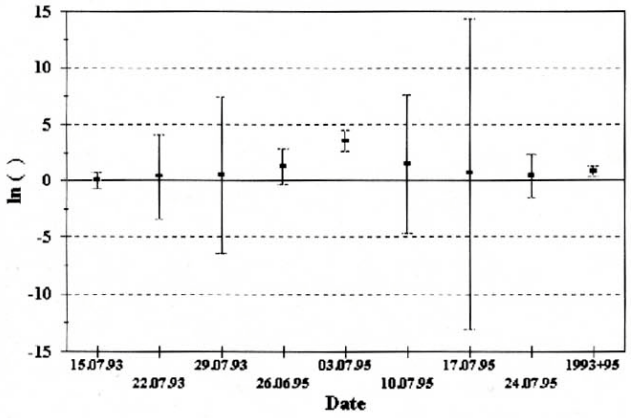

Figure 1: Odds-ratio estimates and the respective confidence intervals for alate / apterous of *Sitobion avenae*.

EXAMPLE 2: *PSILA ROSAE* INFECTED WITH *ENTOMOPHTHORA SCHIZOPHORAE*

Carrot flies were sampled in two successive seasons (1983 and 1984) by use of a sweep-net. At one locality three areas ((1) hedge, (2) carrot field 10 m from the hedge and (3) carrot field 200 m from the hedge) were chosen for sampling of carrot flies. In the hedge sweeping was done to a height of 3 m and in the field the sweeping was done between the carrot rows. Carrot flies were incubated in the laboratory at 17.5°C and mortality was recorded daily for 10 days.

E. schizophorae was the most common fungus infecting carrot flies in the two investigated seasons. It was observed that carrot flies caught in the hedge generally showed a higher prevalence than flies caught 10 m and 200 m away from the hedge (e.g. prevalence in August, 1984 was 56% in hedge, 40% in the field 10 m from the hedge and 25% in the field 200 m from the hedge).

Odds-ratio estimates were calculated to document these differences. The estimates were calculated for each sex separately because of differences in prevalence in relation to sex (data not shown). Weighed common Odds-ratio estimates (ψ^*_{MH}) and corresponding confidence limits are shown in table 2. The tests documented a higher prevalence for both sexes in the hedge than in the field especially the field 200 m away from the hedge.

Table 2: Analysis of the persistence in differences in prevalence of *Entomophthora schizophorae* among *Psila rosae* caught in different sampling areas (Denmark).

	ψ^*_{MH}	Confidence limit (95%) around ψ^*_{MH}	Homo-geneity test
Psila rosae ♂, 1983 & 1984			
Hedge / Carrot field 10 m from hedge	5.32	3.77-7.51	29.1^{NS}
Carrot field 10 m from hedge / Carrot field 200 m from hedge	2.88	1.60-5.17	10.1^{NS}
Psila rosae ♀, 1983 & 1984			
Hedge / Carrot field 10 m from hedge	1.56	1.25-1.95	69.8***
Carrot field 10 m from hedge / Carrot field 200 m from hedge	9.40	5.70-15.49	16.0^{NS}

Homogeneity tests showed that the assumption of common Odds-ratio holds (except for the comparison between females in the hedge and in the field 10 m from hedge). The comparison between hedge and the field (10 m away) gave a weighed common Odds-ratio on 5.32 and 1.56 for males and females respectively (significantly different (.05) from 1).

In contrast to example 1 we suggest that the main reason for differences in prevalence of adult carrot flies caught at different sampling areas can be explained by behaviour of the flies. The adult carrot flies prefer high humidity and stay in the hedge during night and during dry periods of the day. A consequence of this behaviour is that the carrot fly population aggregates in small areas in the hedge where high humidity and high inoculum density is present. Flies with advanced infections will perhaps move to a lesser extent than healthy flies or newly infected flies.

CONCLUSIONS

- → The Odds-ratio (ψ) is a useful estimate to express differences in prevalence in subpopulations within a host-pathogen system.
- → The weighed common Odds-ratio (ψ^*) can statistically document consistent differences (e.g. over time) in prevalence between subpopulations (e.g. morph, sex, sampling area).
- → Thus, the weighed common Odds-ratio (ψ^*) estimates and belonging Chi-test lead to
  - → guiding novel sampling strategies in order to obtain an unbiassed expression of prevalence
  - → designing biological studies in search for explanatory models

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