Double-stranded RNA Elements in the Root Rot Fungus *Heterobasidion annosum*

Katarina Ihrmark
Department of Forest Mycologi and Pathology
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2001

Acta Universitatis Agriculturae Sueciae
Silvestria 210

ISSN 1401-6230
ISBN 91-576-6094-8

Tryck: SLU Service/Repro, Uppsala 2001

Abstract

Ihrmark, K. 2001. *Double-stranded RNA elements in the root rot fungus Heterobasidion annosum.* Doctor's dissertation.
ISSN 1401-6230, ISBN 91-576-6094-8.

This thesis comprises studies concerning the potential of using double-stranded RNA (dsRNA) mycoviruses as biological control agents against *Heterobasidion annosum*, a pathogen that causes root and butt rot in conifers. A large number of *H. annosum* isolates of various origin have been screened for the presence of dsRNA and it was found that dsRNA elements are present in a low proportion of isolates from most forest sites affected by *H. annosum*. The highest proportion, up to 25%, of dsRNA infected isolates were found in localities heavily infected with *H. annosum*. Sequence information indicates that the dsRNA elements in *H. annosum* belong to the virus family Partitiviridae. Studies on transmission of dsRNA in *H. annosum* indicated that horizontal transmission between vegetative cells occurs readily in this species and that anastomoses that allow dsRNA to transfer occur between both homo- and heterokaryotic mycelia, and also between isolates from different intersterility groups. Vertical transmission into spores was less efficient, particularly into conidiospores. It was concluded that conidia are not important for the spread of dsRNA in *H. annosum*, while basidiospores transmit dsRNA efficiently enough to be of importance for the spread of dsRNA. There was little difference in virulence between dsRNA infected and dsRNA free *H. annosum* isolates of the same genotype in virulence tests on ten-day-old seedlings of pine and spruce. Moreover, the virulence of the fungus was not affected when dsRNA was transferred from isolates of the S intersterility group to a P isolate.

In conclusion, the present work indicates that partitivirus-like dsRNA elements cannot be used as biocontrol agents against *H. annosum*, since they do not cause hypovirulence in this species. However, the ease with which dsRNA was transmitted between incompatible isolates shows promise that deleterious mycoviruses have a potential as biocontrol agents in *H. annosum*, although the low transmission rate of dsRNA into conidia poses a potential problem concerning application of such a biocontrol agent.

Key words: Basidiomycota, boreal forest, fungal viruses, biological control, partitiviruses

Author's address: Katarina Ihrmark, Department of Forest Mycology and Pathology, SLU, Box 7026, SE-750 07 Uppsala, Sweden. E-mail: Katarina.Ihrmark@mykopat.slu.se

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Ihrmark, K., Zheng, J., Stenström, E. & Stenlid, J. 2001. Presence of double-stranded RNA in *Heterobasidion annosum*. *Forest pathology* 31. In press.

II. Ihrmark, K., Johannesson, H., Stenström, E. & Stenlid, J. Transmission of double-stranded RNA in *Heterobasidion annosum*. Submitted.

III. Ihrmark, K., Stenström, E. & Stenlid, J. Double-stranded RNA transmission through basidiospores of *Heterobasidion annosum*. Submitted.

IV. Ihrmark, K., Stenström, E. & Stenlid, J. Virulence and double-stranded RNA in *Heterobasidion annosum*. Submitted.

Introduction

Environmental problems associated with the extensive use of chemicals to control plant pathogens have been increasingly recognised during the last 20-30 years. More environmentally friendly ways to control plant pathogens are desirable and several biological agents that can control disease are used in agriculture and forestry today. However, much more work can be done in this area as new agents and practice methods are required to find new ways to protect crops against pests. This thesis evaluates the possibility to exploit mycoviruses as biocontrol agents against the plant pathogen *Heterobasidion annosum* (Fr.) Bref., which causes root and butt rot in conifers and is responsible for considerable economical losses in forestry.

Heterobasidion annosum

The wood inhabiting basidiomycete *Heterobasidion annosum* is a common pathogen in boreal and temperate forests, particularly in managed stands, and causes root and butt rot mainly in conifers (Korhonen & Stenlid, 1998). In Sweden, the main hosts are Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), but a number of other species can also be affected, including deciduous trees and bushes (Korhonen & Stenlid, 1998). Reduced growth rate and decay of trees infected with *H. annosum* are estimated to cost the Swedish forestry at least half a billion SEK a year and the situation is similar in other countries with boreal forests (Bendz-Hellgren *et al.*, 1998). There are therefore ample reasons to try to reduce the negative effects of this fungus.

Forest practices favour the spread of *H. annosum* far beyond the situation in an unmanaged forest (Korhonen *et al.*, 1998). The pathogen readily establishes in fresh wounds created by heavy machinery used in the forest and in stumps created by thinning and felling. Once established, *H. annosum* spreads vegetatively through the root system into neighbouring trees, decaying the valuable base of the trunk and expanding sometimes more than 10 m up the tree (Stenlid & Wästerlund, 1986). The growth rate is reduced in infected trees and weakened trees are more susceptible to wind throw. Young trees are also killed directly by infection (Bendz-Hellgren *et al.*, 1998). The fruit bodies of *H. annosum* often appear on the root system of fallen trees and along the roots of still standing trees. Basidiospores are spread during most of the year and are the main source of inoculum. Conidiospores are also produced, but their importance as inoculum is not clear. (Korhonen & Stenlid, 1998)

Current practices for control

H. annosum does not establish when the temperature drops below five degrees, either because of a lack of spores or inability to grow at low temperatures, or a combination of both. Thus, in Scandinavia, one way to avoid spreading the

pathogen into a healthy stand of trees is to carry out fellings during the colder part of the year (Brandtberg *et al.*, 1996). This is not always economically feasible, but there are alternatives. To prevent new infections from establishing, stump surfaces are usually treated immediately after felling with urea, borate or the biocontrol agent *Phlebiopsis gigantea*, a wood decaying fungus (Thor & Stenlid, 1998). These treatments are effective in stopping new infections by *H. annosum*, but they have no effect on vegetative spread via root contacts in already infected areas and new methods to control *H. annosum* are needed. One possibility would be to find another fungus that can grow through the soil or wood and compete with *H. annosum*, without being as pathogenic as *H. annosum*. Another possibility would be to find viruses that can spread in an already established population of *H. annosum* and slow down or stop the growth of the pathogen in living trees, thus saving valuable timber.

Intersterility groups

H. annosum is a species complex divided into several intersterility (IS) groups, which differ in their host preferences. Three IS groups are recognised in Europe and Asia (Korhonen, 1978; Capretti *et al.*, 1990) and two in North America (Chase & Ullrich, 1988; Harrington *et al.*, 1989) and they are named after their main hosts. The European S group is mainly found on spruce, but in Asia the same IS group is found also on other hosts. Pine is the main host for the European P group, but it is found on a number of other hosts as well. The North American P group is present on pines and sometimes hardwood trees, while the North American S group is found on several different hosts, including *Abies*, *Picea* and *Tsuga*. The third European IS group is called F, after the main host fir. These groups could well be recognised as separate species and species names have been suggested for some of the IS groups by Niemelä and Korhonen (1998). The name *Heterobasidion annosum* is proposed as the name of the European P group, *Heterobasidion parviporum* as the name of the European S group and *Heterobasidion abietinum* as the name of the F group. The intersterility between the groups is not complete and matings are possible in the laboratory at a low rate (Korhonen & Stenlid, 1998).

Mycoviruses

The first mycovirus was found in the edible mushroom *Agaricus bisporus*, which has been cultivated for several hundred years. During the 1940s a disease, termed La France disease, was discovered in cultivations of mushrooms and it was soon clear that the disease was infectious. The disease was suspected to be caused by a virus, but it was more than ten years after the discovery of the disease that Holling, a plant virologist, showed that virus particles were present in diseased isolates. (Buck, 1986)

During the 1960s much interest was directed towards interferon inducing substances. Interferon is produced as an early defence response to virus infections

in animals and in large screening programs for interferon inducers several such substances were found in fungi, *e.g.* in *Penicillium* and *Aspergillus* spp. These fungal substances were subsequently shown to consist of dsRNA and to be of viral origin (Buck, 1986). After the early discoveries of mycoviruses it has become clear that viruses occur commonly in fungi (Buck, 1986; Nuss & Koltin, 1990; Ghabrial, 1994; Ghabrial & Hillman, 1999). One reason for the belated discovery of mycoviruses, compared to viruses of animals, plants and bacteria, is probably that most mycoviruses do not cause any detectable phenotypic changes in their hosts.

Mycoviruses do not have an extracellular phase to their life cycle and are transmitted only by intracellular routes. Because fungi give ample opportunities for viruses to spread intracellularly, by producing a profusion of spores, both sexual and asexual, and through anastomosis at hyphal contacts, mycoviruses have access to highly efficient means for transmission (Buck, 1986; Ghabrial, 1998).

The majority of viruses that have been found in fungi have genomes of dsRNA. Many of them belong to the virus families *Partitiviridae* and *Totiviridae*, have isometric (symmetrical) capsids and are associated with symptomless infections of their hosts (Ghabrial, 1998). Mycoviruses of other compositions have also been reported. There are many reports of mycoviruses from basidiomycetes and ascomycetes, but comparatively little attention has been paid to lower fungi. However, dsRNA mycoviruses have been reported from the zygomycetes *Mucor* spp. (Vagvölgyi *et al.*, 1998) and *Rhizopus* spp. (Papp *et al.*, 2001) and from the chytrid *Allomyces arbuscula* (Khandjian *et al.*, 1977).

Mycovirus taxonomy

In contrast to living cells, which always have genomes of dsDNA, virus genomes can be made up of different types of nucleic acids. This is an important character for classification of viruses. Viral genomes can be made up of DNA or RNA, both of which can be either single-stranded (ss) or double-stranded (ds). ssRNA viruses are further divided into plus and minus, depending on whether the strand is directly coding or not. There are also retroviruses, viruses with DNA or RNA genomes of which a DNA copy is made and inserted into the host genome.

Members of seven virus families and one unassigned genus infect fungi, according to modern viral taxonomy (Van Regenmortel *et al.*, 2000).

Hypoviridae: Lack true virions (virus particles), but pleomorphic (irregular) vesicles surround the genetic material of the viruses. The genome consists of one segment of linear dsRNA, 9-13 kbp in size. Several members of this family are recognised but so far hypoviruses have only been found in *Cryphonectria parasitica*.

Partitiviridae: Virions are isometric and the genome consists of two linear dsRNA segments, usually of similar size. The total genome is 4-6 kbp in size. Plants and

fungi are infected by members of the family and many fungal species have been reported to be infected with partitiviruses.
Totiviridae: Virions are isometric and the genome consists of a single molecule of linear dsRNA, 4-7 kbp in size. Members infect fungi and protozoa and have been reported several times from fungi.
Barnaviridae: The viral particles are bacilliform and the genome consists of a single linear molecule of a positive sense ssRNA, 4.0 kbp in size. Only one member of this family has been reported, from *Agaricus bisporus*. This virus is often found in mushrooms afflicted with La France disease, although it is not the major agent causing that disease.
Narnaviridae: Lack true virions. The genome consists of a single segment of either ssRNA or dsRNA of 2-3 kbp in size. Members of this family have been found in *Saccharomyces cerevisiae* and *Cryphonectria parasitica*. Currently three viruses are assigned to the family.
Metaviridae: Retrotransposons with poorly characterised morphology. The genome consists of positive strand ssRNA, 4-10 kbp in size. Members of the family infect fungi, plants and invertebrates. Four or five members have been found in fungi.
Pseudoviridae: Retrotransposons with poorly characterised morphology. The genome consists of positive sense ssRNA, 5-8 kbp in size. Fungi, plants and invertebrates are infected by these viruses and in fungi, members of the family have been reported from *S. cerevisiae*.
Rhizidiovirus: Virions are isometric and the genome consists of a single molecule of linear dsDNA, 27 kbp in size. Has only been found in *Rhizidiomyces*, a genus belonging to Hyphochytriomycota (Hawksworth *et al.*, 1995), which is closely related to Oomycota and algae in the kingdom Straminipila and is not considered to belong to the kingdom Fungi (Berbee & Taylor, 1999). It is therefore doubtful if *Rhizidiovirus* should be classified as a mycovirus.

There is also a number of recognised mycoviruses that have not been assigned to any family. Most of them have genomes of dsRNA and isometric virions.

Characteristics of fungi of importance for mycovirus transmission

Fungi, like several other groups of organisms, have a cell wall which acts as a barrier for virus penetration. Plant viruses circumvent this barrier by being transmitted by vectors. Bacterial viruses, so called phages, often attach to receptors on the outside of bacterial cells and inject viral material into the cell, leaving the viral coat outside the cell wall. To get out of the cell, phages often lyse the bacteria. It seems that fungal viruses have not solved the problem that the barrier of the cell wall poses. Instead they have adapted to not passing the cell wall and are only transmitted intracellularly.

A special feature of fungi is the ability to anastomose, *i.e.* the ability of hyphae to fuse upon contact. This ability is well developed in ascomycetes and basidiomycetes, while lower fungi have little ability to anastomose (Deacon, 1984). This is an important feature of fungi from the perspective of mycoviruses, since it

makes a completely intracellular existence possible, without spread being limited only to the progeny of the host.

In fungi there are three complementary incompatibility systems: i) One system causes interspecific incompatibility and inhibits anastomoses between hyphae from different species; ii) A second system, the mating system, also referred to as homogenic incompatibility or heterogenic compatibility, makes it possible for unrelated individuals of the same species to anastomose, to recombine and to produce sexual spores. This system is controlled by mating-type (*mt* or *mat*) genes and only individuals with different alleles can mate, and iii) A third system, the somatic, or vegetative, incompatibility system, is controlled by *vic*- or *het*-genes and allows anastomosis between individuals with the same alleles. In *H. annosum* somatic incompatibility is controlled by at least three loci (Hansen *et al.*, 1993). Individuals of the same species but with different *vic* or *het* alleles can often anastomose to some extent, but usually the fused cells are killed shortly after anastomosis. Somatic incompatibility reactions have been reported only for higher fungi, which is not surprising considering that these are the fungi with capacity for anastomosis. In ascomycetes somatic incompatibility is expressed between homokaryons. In basidiomycetes homokaryons with complementary *mat* alleles fuse and produce heterokaryotic secondary mycelia and somatic incompatibility is expressed between heterokaryons. Single hyphae that lose a nucleus and become homokaryotic are again free to fuse with cells with complementary *mat* alleles. Homokaryons can also fuse with hyphae of a heterokaryon and thereby acquire a nucleus. The requirement for and the biological significance of a somatic incompatibility system is unclear, but it acts as a genetic isolation mechanism and is a barrier to cytoplasmic exchange, thus reducing the spread of mycoviruses, debilitated organelles and deleterious plasmids within a fungal population. It may also limit outbreeding in certain fungal species. (Rayner *et al.*, 1984; Leslie, 1993; Begueret *et al.*, 1994; Worrall, 1997; Wu *et al.*, 1998)

Transmission

Numerous attempts to infect fungal mycelia with purified virus extracts have been made but all have been unsuccessful (Buck, 1979, 1986; Lecoq *et al.*, 1979). There are a few reports of successful infection of fungi with purified viruses, but the validity of these successes are doubtful. Two major criticisms of these reports are that either the extracts were not completely free of living cells, that could transmit the virus, or the experiments were conducted in such a way that infected spores or mycelium could reach the uninfected mycelium that was to be infected. For example, a filtered extract from diseased fruit bodies of *Laccaria laccata*, associated with virus infection was watered onto a natural site with only healthy fruit bodies and caused diseased fruit bodies to appear one year later (Blattny & Kralik, 1968). This experiment was conducted outdoors and it is possible that infected spores arrived at the site from diseased fruit bodies further away, that infected mycelium grew into the site or that infected mycelium was present at the site already at the start of the experiment. There have been several reports of

successful infections of *Agaricus bisporus* sporophores with cell-free virus extracts (Hollings, 1962; Hollings *et al.*, 1963; Dieleman-Van Zaayen & Temmink, 1968). Also in these cases it is possible that infected spores have arrived at the experimental sites and infected the mycelium. A few spores could have reached the experiments through the air or the virus extracts. It is also possible that the apparently healthy mycelium had undetectable low virus titers, which later increased and caused symptoms (Ghabrial, 1980; Buck, 1986).

The fungal cell wall is probably responsible for the failure to infect intact mycelia with pure virus extracts. For research purposes the possibility to infect fungi with cell-free virus extracts is desirable and attention has been turned to infecting protoplasts with mycoviruses, rather than intact mycelium. Several of the early attempts to infect protoplasts failed (Lecoq *et al.*, 1979), but successes have also been reported. Protoplasts of *Penicillium stoloniferum* can be infected at a high rate with virus extracts, according to Lhoas (1971). Pallett (1976) got similar results for *Penicillium chrysogenum*, *Marasmius androsaceus* and *Mucor hiemalis*. These reports are exceptional because of the high frequencies of infection reported. Others who have managed to infect protoplasts with virus extracts report very low frequencies of infection, as in *Cryphonectria parasitica* (Van Alfen *et al.*, 1984), *Pleurotus ostreatus* (Van der Lende *et al.*, 1995) and *Helminthosporium victoriae* (Ghabrial, 1986). By using electroporation, protoplasts of several *Cryphonectria* and *Endothia* species have been infected with viral transcripts of hypoviruses (Chen *et al.*, 1994). It is also possible to infect protoplasts by fusing virus-infected protoplasts with virus-free protoplasts, as has been shown for *Pyricularia oryzae* (Lecoq *et al.*, 1979), *Gaeumannomyces graminis* (Stanway & Buck, 1984) and several *Aspergillus* spp. (Varga *et al.*, 1994; Coenen *et al.*, 1997; Van Diepeningen *et al.*, 1998). During mating of yeast cells, the cell wall is partly broken down, creating a kind of natural protoplast. When cells of the yeast *Saccharomyces cerevisiae* mate in a solution with viral particles, the diploid cell can become infected with the virus (El-Sherbeini & Bostian, 1987), indicating that extracellular transmission of mycoviruses is possible during special circumstances. The infection rate was much lower than infection of prepared protoplasts, but it is intriguing that extracellular transmission might be possible.

The efficiency of transmission of mycoviruses between fungal isolates through anastomoses depends to a large extent on the compatibility of the isolates (Buck, 1998). This has been extensively studied in *Cryphonectria parasitica*, where it has been shown that transmission occurs most readily between isolates with the same *vic* genes, and with increasing number of different *vic* genes the transmission efficiency decreases (Liu & Milgroom, 1996). Similar results have been presented for *Ophiostoma ulmi* (Brasier, 1986) and *Aspergillus nidulans* (Coenen *et al.*, 1997). Mycoviruses are transmitted at heterokaryon formation in the basidiomycete *Rhizoctonia solani* between isolates of the same incompatibility group (Finkler *et al.*, 1985) and in the smut fungus *Ustilago maydis* between sexually compatible isolates (Day & Anagnostakis, 1973; Day & Dodds, 1979). In the basidiomycete

A. bisporus the basidiospores are usually heterokaryotic (Elliott, 1985; Summerbell *et al.*, 1989). Although heterokaryon formation is not necessary, mycoviruses are still transmitted at hyphal contacts between genetically similar, but not identical, heterokaryotic isolates of *A. bisporus* (Sonnenberg & Van Griensven, 1991; Sonnenberg *et al.*, 1995).

Transmission of dsRNA into asexual spores is in most cases very efficient (Buck, 1986, 1998), but a few exceptions are known. For example, in *C. parasitica* (Melzer *et al.*, 1997) and *Aspergillus* section *flavi* (Elias & Cotty, 1996) transmission rates range between 0-100%, depending on the virus and host isolate. For basidiomycetes there are no reports of transmission of mycoviruses into conidia, but mycoviruses are efficiently transmitted into the asexual uredospores of rusts (Dickinson *et al.*, 1990; Zhang *et al.*, 1994). Many basidiomycetes are not known to produce conidia, which makes the lack of information about mycoviruses in basidiomycete conidiospores understandable. In contrast to many other basidiomycetes, *H. annosum* do produce conidia and thus offers an opportunity to study virus transfer into conidia in a basidiomycete.

Transmission of dsRNA mycoviruses into ascospores is restricted in many filamentous ascomycetes (Buck, 1986). There is no transmission of cytoplasmic dsRNA hypoviruses into ascospores of *Cryphonectria parasitica* (Anagnostakis, 1988), but a mitochondrial dsRNA element of *C. parasitica* is transmitted to nearly half of the ascospores (Polashock *et al.*, 1997). Low frequencies of transmission of dsRNA into ascospores has been reported in *Aspergillus nidulans* (Coenen *et al.*, 1997), *Gaeumannomyces graminis* (McFadden *et al.*, 1983), *Magnaporthe grisea* (Chun & Lee, 1997) and *Ophiostoma ulmi* (Rogers *et al.*, 1986). High transmission rates of dsRNA into ascospores of filamentous fungi have occasionally been reported, as in *Neosartorya hiratsukae* (Varga *et al.*, 1998). In contrast to most filamentous fungi, transmission of cytoplasmic dsRNA into ascospores of yeasts is very efficient, as in *Saccharomyces cerevisiae* (Brewer & Fangman, 1980).

Efficiency of transmission of mycoviruses through basidiospores has only been studied for a few species, although basidiospores are often considered to spread mycoviruses fairly well (Buck, 1986, 1998; Ghabrial & Hillman, 1999). In *A. bisporus*, La France disease is spread through basidiospores (Schisler *et al.*, 1963; Romaine *et al.*, 1993). An average of 65-75% of viable basidiospores from *A. bisporus* contain dsRNA (Romaine *et al.*, 1993) and infected spores show an increased rate of germination and viability (Schisler *et al.*, 1967; Dieleman-Van Zaayen, 1970; Romaine *et al.*, 1993). A degenerative disease of *Rhizoctonia solani*, associated with the presence of dsRNA (Castanho *et al.*, 1978), is transmitted to basidiospores at a high rate (Castanho & Butler, 1978). In both the smut fungus *Ustilago maydis* (Day & Dodds, 1979) and the yeast *Phaffia rhodozyma* (Pfeiffer *et al.*, 1996) mycoviruses are transmitted to a high proportion of basidiospores. Viruslike particles are transmitted to basidiospores of *Lentinus*

edodes and these particles are probably also dsRNA mycoviruses (Van Zaayen, 1979). In *Agrocybe aegerita* the transmission of dsRNA to basidiospores is weak or non-existent (Barroso & Labarere, 2000), which is contrary to transmission patterns found in other basidiomycetes.

RNA mycoviruses in the chytrid *Allomyces arbuscula* are transmitted through both haploid and diploid zoospores (Roos *et al.*, 1976). There are no reports about transmission of dsRNA mycoviruses into sexual spores of zygomycetes.

There are no known transmission vectors for mycoviruses (Buck, 1986; Ghabrial, 1998). It is difficult to envisage any kind of vector that could transmit mycoviruses, but the possibility cannot be completely ruled out. In plants, viruses can be transmitted by insects and nematodes, as well as by a few chytrids and plasmodiophorans (Brown *et al.*, 1995; Campbell, 1996; Perring *et al.*, 1999). It is possible that there are insects or nematodes that play the same role in fungi, although none has yet been reported.

Effects of dsRNA mycoviruses on host phenotypes

Although many mycoviruses have no apparent effect on their hosts, there are also many reports of mycoviruses that do cause phenotypic changes. Some changes seem to be beneficial for the host, while others more closely resemble diseases. In general a large number of fungal isolates are screened for presence of viruslike particles or dsRNA and the infected isolates are then compared with the uninfected isolates, but fungal and virus strains display too large variation among themselves for this to be a very useful approach. It is only when isogenic strains, virus-free and infected with virus, can be compared that any conclusions can be drawn about small changes in phenotype. If a large proportion of the virus strains in a fungal species cause large changes in the host phenotype, though, it will be easier to associate the mycoviruses with the symptoms, whatever approach is taken to study the connection.

Several yeasts, as well as the smut fungus *U. maydis*, produce killer toxins (Ghabrial, 1994; Magliani *et al.*, 1997). These toxins kill sensitive cells of the same or congeneric species as the producer strains. Unrelated microorganisms can also be susceptible. The cells producing the toxins are themselves immune to their own toxin and some cells without the ability to produce toxins are also immune. The genetics behind the killer toxin systems vary between different systems and there can be several different systems in one species. In some systems the toxin and immunity are encoded by nuclear genes, but this is not always the case. In *Kluyveromyces lactis* toxin and immunity are encoded by linear DNA plasmids. Both toxin and immunity are encoded by dsRNA in some killer toxin systems, *e.g.* in *S. cerevisiae* and *U. maydis*. Sometimes only the toxin is encoded by dsRNA, while immunity is encoded by nuclear genes, *e.g.* in *U. maydis* (Ghabrial, 1994; Magliani *et al.*, 1997). The dsRNAs involved in killer toxin systems are satellites

to dsRNA mycoviruses belonging to the virus family *Totiviridae* (Ghabrial, 1998). In yeast, the killer phenotype offers advantages for the killer yeasts themselves in some environments, but killer strains generally ferment more slowly than non-killer strains. This has practical implications in the wine industry, where killer strains reduce product quality in wine production (Van Vuuren & Jacobs, 1992). Killer strains may also have advantageous effects and carefully selected killer strains or killer resistant strains with desirable fermenting characteristics are used in the fermentation industry (Magliani *et al.*, 1997).

In mushroom cultivation the disease known as die-back or La France disease is of viral nature (Geels *et al.*, 1988). *Agaricus bisporus* is affected, while *A. bitorquis* is resistant to infection, probably due to virus particles not being able to penetrate the cells (because of a lack of anastomoses between *A. bisporus* and *A. bitorquis*) or the virus not being able to multiply in *A. bitorquis*. Infected mycelium grows weakly and patchy and few fruit bodies are produced. Contrary to what the name implies, mycelium and fruit bodies do not actually die, but fruit bodies appear only where the mycelium has managed to grow and can appear later than usual. The sporophores are often abnormal, *e.g.* they stay too small, have long, bent stipes or are greyish-white to brown. Fruit bodies often mature and release spores faster than normally and infected spores germinate faster than uninfected spores, ensuring the spread of the disease. Poorly growing mycelium and deformed fruit bodies are sometimes found in other fungal species, *e.g. Lentinus edodes* (Shen *et al.*, 1993). Virus particles or dsRNA are often found in these isolates, but evidence of an association between mycoviruses and disease are rare. The fruit bodies of *H. annosum* are very variable and it is therefore difficult to isolate viruses in this species by searching for abnormal sporophores.

Mycoviruses in plant pathogenic fungi can affect the virulence of the pathogens. There are many reports of increased virulence (hypervirulence) or reduced virulence (hypovirulence) in isolates infected with dsRNA mycoviruses. Hypervirulence, associated with the presence of certain dsRNA segments, has been found in a few species, *e.g. Nectria radicicola* (Ahn & Lee, 2001). The relationship between dsRNA and virulence is complex in certain species, which explains some of the confusion from early reports. In both *Rhizoctonia solani* (Finkler *et al.*, 1985; Jian *et al.*, 1997) and *Chalara elegans* (Punja, 1995) certain dsRNA elements are associated with hypovirulence, while others show the opposite effect. Mycoviruses causing hypovirulence in plant pathogens are of interest as possible biocontrol agents, although successes in this field are rare. A few of these hypovirulence systems are presented below. No naturally occurring hypovirulent strains of *H. annosum* have been isolated, otherwise it would have been interesting to search for mycoviruses in such isolates.

Cryphonectria parasitica

The hypoviruses of *C. parasitica* are among the most intensely studied mycoviruses. The mycoviruses have been proved beyond doubt to be responsible

for hypovirulence. Isolates of *C. parasitica* have been transformed with cDNA of hypoviruses and the transformants, originally virulent and virus free, show all the symptoms of being hypovirulent, including being infected with hypoviruses (Choi & Nuss, 1992). In the transformants the ascospore progeny also retain the hypovirulent phenotype, which increases the transmission possibilities (Chen *et al.*, 1993; Anagnostakis *et al.*, 1998). The fungus causes chestnut blight which has almost eradicated North America's indigenous chestnuts and threatened to do the same in Europe. The appearance of the hypoviruses has reduced the severity of the epidemic in Europe (Heiniger & Rigling, 1994). Unfortunately, although hypoviruses are present also in North America and European hypoviruses have been released several times, the disease is not held in check in America to the same extent as in Europe, except in a few localities (Anagnostakis, 1987; MacDonald & Fulbright, 1991). The high number of vegetative incompatibility groups of *C. parasitica* in North America is thought to impede the spread of hypoviruses and thus prevent control of the disease (Anagnostakis, 1987; Liu & Milgroom, 1996; Liu *et al.*, 1996). However, with ascospores retaining viruses in transformants, the hypoviruses can be spread to new vegetative incompatibility groups and thus control of the disease could possibly be accomplished (Anagnostakis *et al.*, 1998). When natural spread of hypoviruses is not enough to keep the trees healthy, hypovirulent isolates of *C. parasitica* are used as biocontrol agents of chestnut blight in chestnut orchards in Europe (Heiniger & Rigling, 1994). Blight cankers caused by virulent strains of *C. parasitica* heal when the isolates are converted to the hypovirulent phenotype and the trees survive. Besides hypovirulence other phenotypic changes also occur in the fungus when infected with hypoviruses. *In vitro*, reduced production of pigment (Elliston, 1985), laccase (Rigling *et al.*, 1989), oxalic acid (Vannini *et al.*, 1993) and cryparin (Carpenter *et al.*, 1992) can be observed in infected isolates. Conidial production and spore viability decrease (Peever *et al.*, 2000) and few or no sexual spores are produced (Anagnostakis, 1982, 1988).

Ophiostoma spp.

In *Ophiostoma ulmi* and *O. novo-ulmi*, the two fungi responsible for Dutch elm disease, dsRNA elements known as d-factors exert a deleterious effect on the host (Brasier, 1986). Infected isolates grow irregularly, have reduced spore production, reduced conidial viability, reduced production of the toxin cerato-ulmin and the cytochrome oxidase system is disrupted (Brasier, 1986; Rogers *et al.*, 1987; Sutherland & Brasier, 1995, 1997). Transmission of the d-factors in each species is restricted, but not completely blocked, by vegetative incompatibility (Brasier, 1986). When the pathogen establishes in a new area, few vegetative compatibility (vc) groups are involved and the d-factors present at the site spread rapidly. The number of vc-groups increases rapidly at a newly infected site and the spread of the d-factor is reduced. It has been speculated that the presence of the d-factors drives the increase of vc-groups behind the epidemic front in infected elm stands following initial establishment of single highly virulent genotypes of *O. novo-ulmi* (Brasier, 1990). During the yeast phase of the fungus and production of ascospores the d-

factor is often lost. In the pathogenic phase, yeast growth of the fungus is favoured and the d-factor is easily lost, while in the saprotrophic phase the d-factor is acquired and retained (Webber, 1993). Although the d-factors can prevent an infection from occurring by increasing the number of spores necessary to establish an infection, the d-factors do not manage to control the current Dutch elm disease epidemics (Milgroom, 1999).

Rhizoctonia solani

dsRNA is common in isolates of *R. solani* and the presence of dsRNA has been associated either with hypovirulence or with virulence, but the mere presence or absence does not determine the role of dsRNA in *R. solani* (Tavantzis, 1994). A relatively high degree of genetic heterogeneity exists among dsRNAs in this collective species (Bharathan & Tavantzis, 1990). A disease called Rhizoctonia decline is associated with certain dsRNA elements (Castanho *et al.*, 1978), but only transmissible to genetically identical isolates (Castanho & Butler, 1978). The failure to transmit the disease could also be due to somatic incompatibility between the isolates (Anderson, 1984). The disease is characterised in culture by a reduction in the amount of pigment, reduced growth rate, a low production of sclerotia and an irregular appearance. Virulence is reduced in affected isolates. Another dsRNA element has also been associated with hypovirulence in *R. solani* (Jian *et al.*, 1997) and in field and greenhouse experiments an isolate infected with this dsRNA functioned as a biocontrol agent against *R. solani* in potato (Bandy & Tavantzis, 1990; Tavantzis, 1994).

Sclerotinia spp.

In *S. minor* transmissible hypovirulence, possibly due to dsRNA, can reduce damage caused by the pathogen when a hypovirulent isolate is applied as biocontrol after a virulent isolate has been allowed to establish on lettuce leaves, provided that the isolates are compatible (Melzer & Boland, 1996). Transmissible hypovirulence associated with dsRNA also exists in *S. homoeocarpa* (Zhou & Boland, 1997) and can suppress disease when applied onto turf together with a virulent isolate (Zhou & Boland, 1998). This work is still at the experimental stage, but it might be possible to use transmissible hypovirulence as biocontrol against *Sclerotinia* spp. in the future. At the moment, though, it is unclear if control is achieved by converting virulent isolates to hypovirulent or whether some other mechanism, like induced resistance, is at work (Zhou & Boland, 1998).

Objectives

The main objective of this study was to investigate the possibility of using mycoviruses for biocontrol of *H. annosum*. The thesis consists of several parts (see i-iv) which in different ways contribute to fulfilling the main objective.

i) To find out whether *H. annosum* contains dsRNA mycoviruses, since no viruses were reported previously for this species.

ii) To characterise the dsRNA in *H. annosum* by nucleotide sequencing.

iii) To show transmission of dsRNA in *H. annosum.*

iv) To determine if dsRNA has any effect on virulence in *H. annosum.*

Methods

Several methods can be used to screen for mycoviruses. In all methods there is a risk to overlook viruses, since the concentration of viruses may be lower than the detection level of the technique chosen. To look for virus-like particles in electron microscope was a popular method during the early history of mycovirus research (Buck, 1986), but there are disadvantages, *e.g.*, many normal constituents of the cell can look like viruses. Since most mycoviruses have genomes consisting of dsRNA, it is nowadays more common to screen for the presence of dsRNA instead of scanning for virus-like particles. Antibodies against dsRNA can and have been used in screenings, but today it is more common to extract nucleic acids, separate dsRNA from other nucleic acids and confirm that dsRNA is present in the extracts by enzymatic digestions with nucleases. In the screenings for mycoviruses in Paper I and III the latter method was chosen. dsRNA was separated from other nucleic acids in two different ways, by using cellulose chromatography and LiCl-precipitations. The disadvantage of LiCl-precipitations is that residues of LiCl inhibits reverse transcriptases and this method was therefore avoided for samples destined for sequencing. Basically sequencing was done by making cDNA of dsRNA with random hexamer primers and reverse transcriptase, followed by cloning of cDNA into bacteria and sequencing of the cloned DNA. From the short sequences obtained this way primers could be designed that were used either directly for sequencing or for RACE (rapid amplification of cDNA ends). The products yielded by RACE were subsequently cloned and sequenced.

In Paper II, different combinations of homo- and heterokaryotic isolates of *H. annosum* were paired on agar plates to study transmission of dsRNA. European S and P isolates were included in the study, as well as a North American S isolate. Two *H. annosum* isolates were inoculated on each agar plate, one donor (Si 9 or 95163, both containing dsRNA) and one receptor (no detectable dsRNA). After three months' incubation, subcultures were made of the receptor isolates and from these subcultures single hyphal tips were collected. Several single hyphal tip isolates from each combination were analysed for presence of dsRNA. Since the procedure to extract dsRNA is time consuming, RT-PCR (reverse transcriptase-PCR) protocols were developed to facilitate screening of a large number of isolates for the presence of certain dsRNAs. One set of RT-PCR primers worked well with

dsRNA from several S group isolates and were useful also for sequencing. Several nuclear PCR-RFLP (PCR-restriction fragment length polymorphism) markers were developed to track and identify different *H. annosum* isolates and sequence differences in mitochondrial rDNA useful for the same purpose were identified. All these markers were used to certify transmission of dsRNA. Only when the hyphal tip isolate containing dsRNA also contained the receptor type of mitochondria it was deemed that a transmission of dsRNA had occurred, since mitochondria rarely migrate from one basidiomycete mycelium to another (May & Taylor, 1988; Matsumoto & Fukumasa, 1996; Griffiths, 1996; Barroso & Labarere, 1997). Nuclei, on the other hand, often migrate in basidiomycetes to establish heterokaryotic mycelia, and this migration was followed with the nuclear markers.

Transmission of dsRNA into conidia and basidiospores was investigated in Paper II and III. RT-PCR was used to detect dsRNA in single conidiospore isolates from two *H. annosum* isolates. Conidia were collected from isolates in culture. Circa 100 and 180 single conidial isolates were tested from the two isolates Si 9 and 95163, respectively (Paper II). Basidiospores were collected from fresh fruit bodies in nature. The dsRNAs that potentially infected single basidiospore isolates could not be detected by RT-PCR, since their sequences were not known. Instead the basidiospore isolates were screened by extracting dsRNA, as described above. From each of five fruit bodies, 12-25 single basidiospore isolates were tested for presence of dsRNA (Paper III). RT-PCR is the faster of the two methods, which is why a larger number of conidial isolates than basidiospore isolates could be screened. RT-PCR is also a more sensitive method, detecting dsRNA at lower concentrations than when dsRNA is extracted and visualised directly on gels.

In Paper IV the virulence of dsRNA infected fungal isolates was compared with uninfected isolates. Seedlings of spruce (*Picea abies*) and pine (*Pinus sylvestris*) were grown in individual microcentrifuge tubes, filled with mycelium of *H. annosum*, and placed in propagators. The plants were checked every second day during 3-4 weeks and the proportion of dead plants at each point of time was determined.

Results

To search for dsRNA mycoviruses in *H. annosum*, circa 300 isolates were screened for the presence of dsRNA (Paper I and III). About half of the isolates screened came from the culture collection at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, and had been maintained in culture for up to 13 years, the rest of the isolates were fresh isolates. The isolates had diverse origins, both geographically (Sweden, Norway, Switzerland, Lithuania and Russia) and by isolation technique (conidia, mycelia and fruit bodies), to increase the chance of finding dsRNA. dsRNA was almost exclusively found in isolates that had been isolated as mycelium, either from fruit

bodies or wood (Table 1). In only one case was dsRNA found in an isolate that was isolated as a single conidium from wood. Excluding the 94 isolates from conidia, dsRNA was found in 6%, 14% and 19% of the isolates, in the three screenings that included 110, 59 and 47 isolates, respectively. Results from the three screenings are combined in table 1. Geographically, dsRNA was found from all places where a high enough number of isolates were included in the screenings. None of the four *H. araucariae* isolates included in the first screening contained dsRNA.

Table 1. Number of *H. annosum* isolates from different isolation sources and number of isolates from each isolation source that contained dsRNA

Isolation source	Number examined	Number of isolates containing dsRNA
Mycelium, fruit bodies	51	9
Mycelium, decayed wood	165	15
Conidia	94	1

The dsRNA elements found in *H. annosum* ranged between 1.8 and 2.4 kbp in size. In most isolates, two dsRNA elements were present, always one fragment at a higher concentration than the other.

In Paper I, a partial sequence of dsRNA from *H. annosum* isolate 95163, from Russia, is presented. This sequence shows strong homology to reported sequences of RNA dependent RNA polymerases (RDRP) from partitiviruses from *Rhizoctonia solani*, *Atkinsonella hypoxylon* and *Fusarium poae*. I have partial sequences of dsRNA from eleven other S group isolates from Sweden and Norway. These nucleotide sequences all show strong homologies with each other (Fig. 1). I also have the complete sequence of a dsRNA from a Swedish P group isolate. This isolate seems to contain only one dsRNA element, which again show strong homology to RDRPs from partitiviruses. The P group isolate dsRNA, however, differ considerably from the S group isolate dsRNAs (Fig. 2).

Papers II and III deal with transmission of dsRNA in *H. annosum*. Hyphal anastomosis appears to be an efficient way for dsRNA to spread. In almost all combinations tested, dsRNA was transmitted and somatic incompatibility between heterokaryotic isolates did not seem to stop transmission. dsRNA was transmitted even between isolates of different IS groups. When transmission frequencies of dsRNA to hyphal tips of the two donor isolates, 95163 and Si 9, were checked, a large difference was found between the two isolates. 18% and 91%, respectively, of the single hyphal tip isolates contained dsRNA. Obviously there was a risk to miss transmissions by analysing single hyphal tip isolates, but this was preferred to the risk of analysing a mixture of hyphae from two isolates. Since RT-PCR is a very sensitive method, even a small amount of hyphae from the donor isolate could have given a false result of transmission. In a preliminary study of transmission, recipient isolates were tested for presence of dsRNA without isolating single hyphal tips and the transmission frequency was higher (almost 100%) in this study than when single hyphal tip isolates were analysed.

```
95163 dsRNA   GTTACGAAACCTTCACCGGCGGATGGTTCCGACTCTATCGGACTCTCCAC
Fr110B dsRNA   CTACGAAACCTTCACTGGTGGCTGGCTCCGCTTAAATGCAGAACTGCAC
Si9 dsRNA             GTTTACCGGTGGATGGATGCGCCTCAACGCCGCACTGTTC
87-250 dsRNA                                        ACGCTGCTCTTTTC

95163 dsRNA   ACTCCCGGCGAAAACGTCACCTACATCACGATTGATTGGTCTCGATTTGA
Fr110B dsRNA  AATGGTCTTATTCGAAGTTCCTTTCTTACAACAGATTGGAAACGCTTCGA
Si9 dsRNA     TCTGGATACATGAAAAGGTCTTTTCTGACCTTAGATTGGAAACGATTCGA
87-250 dsRNA  TCTGGTTACATTCGACGGTCTTTCCTGACCCTAGATTGGAAACGTTTCGA

95163 dsRNA   CAAACGCGCATACTTCTGGCTCATCCGGAAGATATTCATCCGCACCCGGC
Fr110B dsRNA  CAAGCGCGCCTATTTTAGTCTGATTCATAGAATCATGGCCATCATTCGCA
Si9 dsRNA     CAAACGAGCTTACTTCTCGCTTATACGCAGAATCATGCTGAATGTTCGAA
87-250 dsRNA  CAAACGCGCTTACTTCTCCCTCATACGAAGGATCATGTTAAACGTTCGAA

95163 dsRNA   AATTCTTTGATTTCGAAAACGGCTACACTCCTACTAAGGACTACCCC---
Fr110B dsRNA  CCTATCTTGACTTCAACAATGGTTATTTACCAAACGAGCTCTACCCTGAC
Si9 dsRNA     CTTATCTCGATTTCAACAACGGATACTTACCTAACATCAATTACCAAGAT
87-250 dsRNA  CCTACCTGGATTTCGAAAACGGATATCTACCTAACATCAACTACCCTGAT

95163 dsRNA   -TTTAGCCCAAC--ACATCCCGACAAG---------CTCCAAGCTCTCTG
Fr110B dsRNA  ACTCGCTCCGAATGGAGCAGTGACAAAGCTCAACGTTTGGAGTTCCTATT
Si9 dsRNA     ACCAAATCAGACTGGAACCACAACAAAGCAATCAAACTCGAACGACTCTG
87-250 dsRNA  ACCAAATCAGACTGGAACCACAACAAGGCACTCAAACTTGAACGCCTCTG

95163 dsRNA   GGAATGGACTATCGAAGCCTTCTTCGACTCCCCGATCGTACTACCAAACG
Fr110B dsRNA  CCTATGGACACTCGAAAACCTCTTTCAAGCGCCAATCGTCTTACCAGACG
Si9 dsRNA     GCTCTGGACGCTGGAGAATCTCTTCAACGCACCAATCGTCCTACCCGACG
87-250 dsRNA  GCTCTGGACGCTTGAAAACCTTTTCAACGCGCCAATCGTCTTACCCGACG

95163 dsRNA   GACACGTCTACAAACGCCGTTTCGCTGGCATCCCATCTGGATTATTCATC
Fr110B dsRNA  GACGCATGTATAGACGACGTTTCGCCGGAATCCCTTCTGGCCTGTTCATC
Si9 dsRNA     GCCGAATGTTCAAACGACGCTACGCTGGAATACCATCTGGACTATTCATC
87-250 dsRNA  GCCGGATGTACAAGCGCCGCTACGCTGGTATACCATCTGGACTATTCATC

95163 dsRNA   ACTCAATTGATGGACTCCTGGTACAACTATGTCATGCTATGCTCCATCTT
Fr110B dsRNA  ACGCAACTCCTCGACTCCTGGTATAACTACACCATGTTAGCGACCATCCT
Si9 dsRNA     ACTCAACTTCTGGATAGCTGGTACAACTACACGATGTTGGCAACACTACT
87-250 dsRNA  ACTCAATTATTGGATAGCTGGTACAATTACACGATGTTGGCAACACTACT

95163 dsRNA   ACACTACATGGACATCGATCCCAAAACTTGCATTATCAAGGTACAAGGCG
Fr110B dsRNA  CTCCGCTCTCGGCTTCAACCCCAAGAGCTGCATCATCAAAGTCCAAGGCG
Si9 dsRNA     ATCTGCAATGGGCTACGACCCAAAGCAGTGCATCATCAAAGTCCAAGGCG
87-250 dsRNA  ATCTGCAATGGGCTACGACCCAAAGCAGTGCATCATCAAGGT

95163 dsRNA   ACGACTCAATCATCCGTCTTGCTATCCTCATCCCCAAAGATGAGCATGAT
Fr110B dsRNA  ATGACTCAATCATCCGTCTTTATGTTTTGATACCTCCTCATTCTCACGAA
Si9 dsRNA     ATGACTCAATCATTCGCTTAGGCATCTTGATCCCCCCTGACTCACACGAA
87-250 dsRNA

95163 dsRNA   AATTTTCTCGCTACTATGCAAACCGTGGCTGACCACCTATTCGGTGCAAT
Fr110B dsRNA  GAATTTTTGCTTAAAATGCAAGAAATCGCTGATTATTACTTCAAATCCGT
Si9 dsRNA     GCATTTTTGCTCAGGATGTCAGAACTCGCCGACTACTACTTCAAATCAGT
87-250 dsRNA

95163 dsRNA   CATCTCCATGGACAAGTCTGAACTACGCAACTCAATCGAAAACTGCGAAG
Fr110B dsRNA  GATCTCGACCCAAAAGTCCGAGATCGGGAACCGCCTTAACGGGCGTGAAG
Si9 dsRNA     CATTTCAAAGGAGAAAAGCGAAATCGCTAACTCCCTCAATGGCCGTGAAG
87-250 dsRNA

95163 dsRNA   TTCTAAGCTATCGACACTTTATGGGTCTGCCTTACCGCGACCTACTCAAG
Fr110B dsRNA  TCCTGAGTTATCGGAATAACAACGGCCAACCATACCGTGACGAAATCCTG
Si9 dsRNA     TGCTCAGTTACCGGCATAACAANGGATTACCCTACAGAGATGAAATCTTG
87-250 dsRNA
```

Fig. 1. Aligned partial sequences of dsRNA from four S group isolates. Fungal isolate names at left-hand side of figure. Origin of isolates: 95163, Russia; Fr 110B, Frossarbo, Sweden; Si 9, Siarö, Sweden; 87-250, Norway

```
H.a. S dsRNA
H.a. P dsRNA  MPHYQIPPY--DIPPVVSD--------VNFDTESFQPHEKYREL-LETYR
A.h. virus    M-----STLL----------IPQDTIAHTFD----EAVASESNLRIDEVP
R.s. virus    M-LYNFKAFISKVSHSIRDYLTEAYIRRNFEWSNFNKLHTDPSTAYYDIR
F.p. virus    M-L-----------ANIRDYFHEKLTRLLYDHKIFQSNSKDPDLTLEAHH

H.a. S dsRNA
H.a. P dsRNA  LSSTDGNLKAIQ----------DFVSEYD----------------FQYFM
A.h. virus    ENYLER--------FIHPSEPENFE---------------------FYS
R.s. virus    DPSNVRAYHAL-FGTRRQPPPKQIE--LESYMLNLIHRNAIKNRPFQFYT
F.p. virus    SSDIERIYKSIHYDFNRSPAPIDYEAQYQSIKHILEDKQSQQGFPHEYYR

H.a. S dsRNA
H.a. P dsRNA  PTGIET-LPDNRVPAPGIRALPQFRFHPVAA----------------LNR
A.h. virus    --LRDSDIPSKRIPKNGIQVFENLKYHTNS--------KDNLYKDQ----
R.s. virus    DLFTDTDLPTDRIPKPGIELV-NAYFHPGNVIRPNPEFSKQLTPDMELDQ
F.p. virus    --LHESPIPDDRIPPSGIKLL-PFEYKSMNVVTATPEVPE----------

H.a. S dsRNA
H.a. P dsRNA  FRSIPRTGFN----ALWTIVNLLATSFMSYVYVISDYCRPAGNIDAIFEN
A.h. virus    -----PSS---GPSPMRGVANIIREYFPQYLDDLRTWCRPKSSDDSIFND
R.s. virus    FESYIPGDIDYGPPIDNELLTLIYRKYPQYLDPITKYCRPAGTTDATFRD
F.p. virus    ------SGFKIHPRIER----LLRSKYPQYLQYVRKYTRPLGTTNATVSD

H.a. S dsRNA
H.a. P dsRNA  FNQAVSPVKDVTSQRLNEIMILIFHFLPIVPHPPVNFPDLRFYKWSLVTS
A.h. virus    FNHEQRITQPFTEERERRLLPLIDHFLGIKPYDIVHYCDTRFYPWKLSTR
R.s. virus    FNKEQIKTEDSDALRLSNIMDLIHEFMNITPYMPLHFVDTFYCKLPLVTG
F.p. virus    FFKPQTPSQPVEPTRINHVMSHVMKKMAITPYLPLHFVDTQYDKRPLANG

H.a. S dsRNA
H.a. P dsRNA  ADYHAHHSSDMQNESTRYWTHLRDTSQLEDRFDYSKNPRSKGFFFNSLLL
A.h. virus    ADYFHNHSRDR-----------KAHAAKSHPD-FATGPTKKSYFINSHLF
R.s. virus    TGYHNRHSYFR-----------RSFAHFCHPELYAEKPTSKGYFFNATKH
F.p. virus    TGYHNRRSHEM-----------NIHALFSHPKEYESKRTSKGYYVNAFLE

H.a. S dsRNA                                          FPMLLHIKTAVVK
H.a. P dsRNA  RCRRIVHNIKYTGWPIPHDPSESKTSFLQRMLYWTLMNPTVMYVRSQISK
A.h. virus    FDRSTVHNIKEYGFPFRPTTDSARNETLLD--LWFKKVPTELLVRSHISK
R.s. virus    ENRFLVHKIKHSGYPFDFTSDSDRNSKMAD--F-LKSFPTMMFTRNHISK
F.p. virus    SARSLIHWIKLYGNPFR-HCPSDLAQSLRE--F-FLQRPTMLFTRNHISD

H.a. S dsRNA  FDAPNKLRTIWGVSKLWIISEAQIYWEYIAWIKLNPGITPMLWGYETFTG
H.a. P dsRNA  ITK-LKVRPVYNAPMLFLMLEAMLTLGLMAQCRKPDNC--ILWSYETIRG
A.h. virus    RDN-LKVRPVYNAPMIYIRIECMLFYPLLAQARKRDCC--IMYGLETIRG
R.s. virus    RDGTLKVRPVYAVDELFLDLECMLAFPATVQARKPECC--IMYGLETIRG
F.p. virus    RDGILKQRPVYAVDDLFLTIESMLTFPAHVIARKPECC--IMYGLETIRG

H.a. S dsRNA  GWFRLYRTLHTPGENVTYITIDWSRFDKRAYFWLIRKIFIR-TRQFFDFE
H.a. P dsRNA  GMHELHRI---STEFNVFMGFDYSRFDQLAPFTIIYHFWATFIPMIIRVD
A.h. virus    GMNELERI---SNAFNSFLLIDWSRFDHLAPFTISNFFFKKWLPTKILID
R.s. virus    SNIKLDSL---AQGFISFATIDWSGYDQRLPWFIVRAFFFIYLPSLLIIS
F.p. virus    SNQILDKI---ASDYKSFFTIDWSGFDQRLPWVIVKLFFTEYIPRLLVVN

H.a. S dsRNA  NGYTPTKDYP-----FS---------------PTHPDKLQALWEWTIEAF
H.a. P dsRNA  RGYQQSTIYTKDQYAYDFEKKYDNLDKSEPKFQEFAKKSNNLFPKHVVAF
A.h. virus    HGYAQISNYHDHVHSFSAQAQSHGIPMISKEYQTPP--EATVFAKK----
R.s. virus    HGYMPTSEYED---------------------TSMN--ISDMFTR-----
F.p. virus    HGYAPTYEYPS--------------------YPDLT--TNDMVSR-----

H.a. S dsRNA  ---------------FDSPIVLPNGHVYKRRFAGIPSGLFITQLMDSWYN
H.a. P dsRNA  SFVIFNILSFIWWWYVFMVFITPDGYGYVRLLAGVPSGIFMTQILDSFVN
A.h. virus    ---VLNLISFLERWYRDMVFVTPDGFAYRRTHAGVPSGILMTQFIDSFVN
R.s. virus    ---FFNLINFTATWYFNMVFLSADGFAFRRQFAGVPSGMLLTQFLDSFGN
F.p. virus    ---LTNLITFLATWYFNMVFVTADGFSYVREHAGVPSGMLNTQFLDSFGN

H.a. S dsRNA  -YVMLCSILHY--MDIDPKTCIIKVQGDDSIIRLAILIPKDEHDNFLATM
H.a. P dsRNA  LFIFVDALLEFGFSIDQIKCFRLFIQGDDNLVFYLGDLTRIFD--FYEWY
A.h. virus    LTILLDGLIEFGFTDEEIKQLLVFIMGDDNVIFTPWTLLKLIE--FFDWF
R.s. virus    LYLIIDSLLEFGCTYDDIKSLMLFIMGDDNSIFTNWTIDKLHD--FISFM
F.p. virus    LFLLIDGLIEFGSTDAEIDDILLFIMGDDNSAFTTWSITHLEQ--FVSFF
```

```
H.a. S dsRNA  QTVADHLFGAIISMDKSELRNSIENCEVLSYRHFMGLPYRDLLKMLAQF-
H.a. P dsRNA  PEYALDRWHMIVSPDKSWITRLRTKIEVLGYTNANGMPHRDVSKLIATLA
A.h. virus    AKYTLDRFGMVINISKSAVTSIRRKIEVLGYTNNYGFPTRSISKLVGQLA
R.s. virus    ERYCLKRWNMHLSKTKSVITTLRSKIETLSYRCNFGKPRRDVEKLIAQLV
F.p. virus    ETYALSRYGMVLSKTKSIITTLRHKIETLSYQCNFGHPRRPIGKLVAQLC

H.a. S dsRNA  YHTKARDPTPE--ITMAQAVGFAYAACGNDYRIHALLEDIYMFY------
H.a. P dsRNA  YPERTILDKNRYPILMSRAIGIAYANAGHDHAVHQLC---YHAYLDARKK
A.h. virus    YPERHVTDA----DMCMRAIGFAYASCAQSETFHALCKKVFQYYFAKT--
R.s. virus    YPEHGLKPQ----FMSSRAIGLAYASCAQDSTFHEFCHDVYRLYLPV---
F.p. virus    FPERGPRPK----YMSARAVGMAWASCGQDKTFHDFCRDVYHEFNDDR--

H.a. S dsRNA  ------KLQGYEPNPAGMSL--VFGNSPDR--PELPFNFDHFPSQNEVQQ
H.a. P dsRNA  SNLSASELKEIIIPYQKLGFYEIFSVNIEELFPTLVRNLDRFPTFYEIRD
A.h. virus    SINERLILKGRKAELPGM-------FFAYPDVSEHIR-LDHFPSLSEVRI
R.s. virus    ADLSPAAIRNTRVWILKL--------LEMEET-EALIP-LDHFPTMSEIQH
F.p. virus    ADLDESAYLHIQSHLPGY-------LKIDESVRQIVD-FQVFPSQQTVYH

H.a. S dsRNA  YFVSLDY-K
H.a. P dsRNA  NLSKWHGPHDIYPMWPL-QFTDRPDFINPDETPITLYDFMTKINLHIP-L
A.h. virus    LLSKFQGYLKETP------FGTIPTFSTPQ-----------------TL
R.s. virus    LLSYYHGPLRPEPKWNYAHFPQDPDFRPKDY--VTLLDYMERNNISFPEL
F.p. virus    TVSRWKGPLSYQPEMGSCSLCQPT

H.a. S dsRNA
H.a. P dsRNA  HHDVL
A.h. virus    RDQTQ
R.s. virus    INFTV
F.p. virus
```

Fig. 2. Aligned amino acid sequences of partitivirus RNA dependent RNA polymerases. One S and one P group *H. annosum* dsRNA, from isolates 95163 and Ör 17-II, respectively, are included. The other three RDRPs have been accessed from Genbank and can be found under accession numbers: L39125, from *Atkinsonella hypoxylon* (A.h.) partitivirus; AF133290, from *Rhizoctonia solani* (R.s.) 717 partitivirus; AF047013, from *Fusarium poae* (F.p.) virus 1

Transmission frequencies into spores varied between isolates. On average, 40% of single basidiospore isolates originating from fruit bodies infected with dsRNA contained dsRNA, but the difference between fruit bodies was large, 10-84%. The proportion of conidia that contained dsRNA was lower. Three and 55% of single conidial isolates contained dsRNA, respectively, where the number 55% refers to isolate Si 9, with a higher concentration of dsRNA than isolate 95163.

No obvious changes in morphology, such as irregular growth or colour changes, have been noted in isolates containing dsRNA. In Paper III, the germination rate of basidiospores from fruit bodies containing dsRNA was compared with the germination rate of basidiospores from fruit bodies with no detectable dsRNA. On average, 34% and 78% of the basidiospores germinated in liquid malt extract medium, respectively, and this difference was significant ($P<0.05$), although there were big differences between individual fruit bodies. Whether presence of dsRNA affect the virulence of *H. annosum* was also tested and the results are presented in Paper IV. Virulence was tested on ten days old seedlings, S group isolates on spruce (*Picea abies*) and P group isolates on pine (*Pinus sylvestris*). Two dsRNAs originating from S group isolates were tested, both in their original isolates and in a P isolate. There was a trend of increased virulence by the two S isolates, Si 9 and

95163, when infected with the dsRNA, vB, originally found in isolate 95163. The dsRNA originally found in isolate Si 9, vH, had no effect on virulence. None of the dsRNAs affected virulence when transferred to a P isolate.

Discussion

Mycoviruses in *Heterobasidion annosum*

dsRNA mycoviruses are ubiquitous in fungi (Buck, 1986; Nuss & Koltin, 1990; Ghabrial, 1994; Ghabrial & Hillman, 1999) and it is therefore not surprising that dsRNA elements are also present in *H. annosum*. These dsRNA elements show characteristics similar to members of the virus family *Partitiviridae*. Partitiviruses are typically isometric and have bipartite genomes, with two segments of similar size and with a total genome length of 4-6 kbp, where one segment codes for an RDRP and the other segment codes for a coat protein (Ghabrial & Hillman, 1999). Several of the dsRNA elements found in *H. annosum* have two dsRNA fragments, where one fragment is present in a much lower concentration. It is possible that many of the isolates with apparently only one dsRNA fragment may in reality have two or more fragments with only one fragment in high enough concentration to be detectable on an agarose gel. There could also be several dsRNA fragments present of the same size and thus indistinguishable on a gel. All detected dsRNA fragments are about 2 kbp, which would give a total genome length of about 4 kbp when two dsRNAs are present, if both fragments belong to one virus. All sequence information of the dsRNAs found in *H. annosum* indicate homology to RDRPs of partitiviruses, especially fungal partitiviruses. An RDRP is a necessity for replication of an RNA virus and the coding of such an enzyme indicates a self-replicating entity. All this taken together strongly indicates that the dsRNA elements found in *H. annosum* are partitiviruses.

Few fungal partitiviruses have been sequenced and sequence information of partitiviruses from only five fungal species have been submitted to public databases so far. Besides sequence information of *H. annosum* partitivirus, sequences of partitiviruses from *Rhizoctonia solani*, *Atkinsonella hypoxylon*, *Fusarium poae* and *Discula destructiva* are available in Genbank. There are considerable differences in sequence of these partitiviruses and similarly the sequences of dsRNA elements from S and P group isolates of *H. annosum* differ considerably (Fig. 2). Interestingly, there is little sequence difference between the dsRNA elements from the S group (Fig. 1) and alignments can also be done on the nucleotide sequences, not only the amino acid sequences as for partitiviruses from different species.

In the screenings for dsRNA in Paper I and III the proportion of dsRNA infected isolates varied between 6-19%. The later screenings revealed a higher proportion of

infected isolates than the first screening. This could reflect an increasing familiarity with extraction procedures, rather than a real difference. There is always a possibility that infected isolates will be overlooked due to dsRNA concentrations below the detection level. The extraction procedure has been improved with time and I assume this can have influenced the amount of infections detected. Another, probably more significant explanation to the difference in the proportion of dsRNA infected isolates is the source of the isolates. In the first screening (6% infected isolates) the isolates had as diverse origin as possible (Paper I), while the higher proportion of infected isolates (14 and 19%) in later screenings (Paper III) came from isolates of more narrow origins. The majority of isolates containing dsRNA in Paper III came from Siarö and Lunsen, Sweden, two areas heavily infected with *H. annosum*. The high concentration of *H. annosum* in one place might facilitate spread of dsRNA in an area through hyphal contacts and increase the chance to find isolates containing dsRNA.

Transmission of dsRNA

A surprisingly low proportion of *H. annosum* conidia contained dsRNA, considering the high proportions of infected conidia that have been reported for other fungal species (Buck, 1986, 1998). In isolate Si 9, where the concentration of dsRNA was higher than in most other isolates, 55% of the germinated conidia contained dsRNA, while in isolate 95163 only 3% of the germinated conidia contained dsRNA (Paper II). Most isolates of *H. annosum* infected with dsRNA contained roughly the same concentration of dsRNA as isolate 95163 and if transmission of dsRNA into conidia reflects the concentration of dsRNA in mycelium, then most isolates will have a low proportion only of their conidia infected with dsRNA. Conidia are therefore probably not important for transmission of dsRNA in *H. annosum*. More information concerning the transmission of dsRNA into conidiospores is needed before it is possible to say if this is true for other basidiomycetes as well. By contrast, in ascomycetes the transmission of mycoviruses through conidiospores is generally efficient (Buck, 1998).

The importance of conidia in the life cycle of *H. annosum* is not known (Korhonen & Stenlid, 1998). In contrast, basidiospores are an important source of inoculum for long distance spread and establishment of *H. annosum*, while mycelium spreads the fungus by vegetative growth to nearby trees. Between 10 and 84% of germinated basidiospores were infected with dsRNA (Paper III), which potentially makes basidiospores more important as transmitters of dsRNA than conidia in *H. annosum*. This suggestion is supported by the fact that basidiospores are also of greater importance than conidia for the spread of *H. annosum*. Considering the ease with which dsRNA was spread through anastomoses in *H. annosum*, hyphal contacts during vegetative growth are probably also an important transmission route. In conclusion, the important ways for spread of *H. annosum*, basidiospores

and vegetative growth, seem also to be important for spread of dsRNA in this fungus.

Somatic incompatibility does not seem to be as important as a barrier against transmission of mycoviruses in *H. annosum* as one could believe, considering the lack of transmission of mycoviruses between incompatible isolates in other fungi (Buck, 1998). Again, these results stem mainly from ascomycetes, where somatic incompatibility is expressed between homokaryotic mycelia, while in basidiomycetes somatic incompatibility is usually expressed between heterokaryotic mycelia (Rayner *et al.*, 1984). In *H. annosum* there are always a number of homokaryotic hyphae, also in a heterokaryon, free to anastomose with other hyphae to acquire a second nucleus, and this is probably why dsRNA was so easily transmitted even between heterokaryotic mycelia (Paper II). Other basidiomycetes can behave in a similar way and thus somatic incompatibility would not be such an important barrier to the spread of mycoviruses in basidiomycetes as it is in ascomycetes. At least, when spread of mycoviruses is discussed, the differences between the two groups must be taken into consideration, which is rarely done at the moment.

Effects of dsRNA

Effects on virulence by the presence of mycoviruses have been studied in a number of plant pathogens, but few of these studies have involved basidiomycetes. To my knowledge, the only basidiomycetes in which the relationship between mycoviruses and virulence of the host has been studied are *R. solani*, *U. maydis* and *Melampsora lini*. In *R. solani* different dsRNA elements can either increase or decrease virulence (Finkler *et al.*, 1985; Jian *et al.*, 1997). In *U. maydis* dsRNA codes for killer toxins, but these toxins are not produced *in planta* and have no effect on virulence (Nuss & Koltin, 1990). In the rust *M. lini* there is no effect on virulence by dsRNA (Pryor *et al.*, 1990). My results indicate that virulence is not reduced by dsRNA in *H. annosum*, but that virulence might be increased by infection of dsRNA. Most mycoviruses seem not to affect virulence (Ghabrial, 1998), but most of the available information stem from studies of ascomycetes and information from more species of basidiomycetes is needed before it is possible to make any general statement concerning the effect of mycoviruses upon virulence in basidiomycetes.

One effect of dsRNA in *H. annosum* was a reduction in the proportion of germinated basidiospores (Paper III), but this apparent effect is tentative and need to be confirmed in isogenic strains, with and without dsRNA, of *H. annosum*. So far the isolates that are available have not produced any fruit bodies, so this comparison cannot yet be done. A reduction in germinability of basidiospores would be negative for the fungus and selection pressure ought to have eliminated any mycoviruses with this effect. The intracellular mode of existence makes

mycoviruses very dependent of their hosts and they can therefore not afford to be too deleterious to their hosts.

Highly virulent viruses have been found only in a few fungal species, e.g. *A. bisporus*, *C. parasitica* and *O. novo-ulmi*. All these species share the common feature that somewhere there exists populations with low diversity of vc-groups. The cultivated mushroom *A. bisporus* is an extreme case, with dense populations of monocultures where viruses have perfect conditions for horizontal transmission between mycelia without any incompatibility barriers. *C. parasitica* and *O. novo-ulmi* have with human help established in areas where they formerly were not present, *i.e.* in Europe and North America. Consequently, populations with low diversity of vc types have appeared in some localities in these areas, since a limited number of individual genotypes were involved in founding these populations, while the diversity of vc types is much higher in the pathogens' natural range. It has been hypothesised that in systems with high diversity of vc types, restriction of horizontal transmission will select for reduced virulence in mycoviruses, while in systems with low diversity in vc types, where vegetative incompatibility have a limited effect in preventing horizontal transmission, there will be a selection for more virulent mycoviruses (Milgroom, 1999). If that is the case, mycoviruses with potential as biocontrol agents will primarily be found in populations with little or no diversity of vc types. Although the spread of *H. annosum* has been favoured by modern forestry, the fungus is still mainly within its natural distribution and the diversity of somatic incompatibility types is high (Hansen *et al.*, 1993; Swedjemark & Stenlid, 1993). This could mean that selection pressures disfavour virulent viruses in *H. annosum*. Alternatively, since somatic incompatibility does not seem to be an important barrier against horizontal transmission of dsRNA in *H. annosum*, the hypothesis might not be valid in this system.

Potential for biocontrol

Mycoviruses are clearly fairly common in *H. annosum*. Can they be used for biocontrol? Mycoviruses are attractive as biocontrol agents since transmission between species is restricted and other organisms than the target species are unlikely to be affected. A mycoviral biocontrol agent can establish in a host population and maintain its own replication without further need for application. However, this is not likely to happen, particularly not if fungal fitness is reduced and transmission is slow. Only a few examples of naturally maintained biocontrol of mycoviruses are known, *e.g.* hypoviruses in *Cryphonectria parasitica*. In *H. annosum* application is not a major concern, since treatments of stump surfaces are standard practices. Spores of *Phlebiopsis gigantea* is applied on stumps immediately in connection with felling and probably dsRNA infected *H. annosum* spores could be applied in the same manner. A possible problem is that *H. annosum* produces conidia whereas *P. gigantea* produces thick walled oidiospores that are more resilient than the conidia of *H. annosum*.

Lack of horizontal transmission seems to be no obstacle against using mycoviruses as biocontrol of *H. annosum*, given the high transmission rate of dsRNA between isolates (Paper II). More problematic is the low proportion of dsRNA infected conidia, since conidia is probably the easiest way to apply dsRNA as a biocontrol agent against *H. annosum*. Besides the difficulty in finding a mycovirus that reduces virulence, for this hypothetical mycovirus to actually be possible to use as a biocontrol agent it must also be transmitted into conidia at a high rate.

Since no reduction of virulence caused by dsRNA could be shown in Paper IV, the work presented in this thesis gives little indication that mycoviruses can be used for biocontrol of *H. annosum*.

Conclusions

i and ii) dsRNA elements, probably belonging to the virus family *Partitiviridae*, infect and can be found in *H. annosum*.

iii) Transmission of dsRNA in *H. annosum* occurs mainly through hyphal contacts and basidiospores. More rarely conidiospores may also transmit dsRNA.

iv) Partitivirus-like dsRNA elements in *H. annosum* do not reduce virulence of this tree pathogen.

The main question in this thesis was whether mycoviruses can be used as biocontrol of *H. annosum*. The results presented here show that dsRNA elements infect *H. annosum*, but that partitivirus-like dsRNA elements are unlikely candidates for biocontrol of *H. annosum*. Although dsRNA is easily transmitted between isolates, thereby allowing for the development of application systems, these partitivirus-like dsRNA elements do not reduce virulence and can therefore not be used for biocontrol.

Future perspectives

There are still unexplored possibilities for mycoviruses as biological control of *H. annosum*, even though the results presented in this thesis are not encouraging.

Further screenings for mycoviruses could reveal more interesting viruses than the ones I have found, viruses that reduce virulence or affect other important traits, like growth rate. Other mycoviruses that infect *H. annosum* than partitiviruses may very well exist, although less frequently than the partitiviruses I have found.

Instead of screening randomly for mycoviruses, a concerted effort to find hypovirulent isolates of *H. annosum* could be made to see if mycoviruses play a role in hypovirulence.

There is also the possibility genetically to modify mycoviruses in order to reduce the virulence of their host, *i.e.* use the viruses as carriers of genetic information rather than rely on the inherent traits of the viruses. The feasibility of this method is doubtful for several reasons. It is much easier to speak of genetic engineering than actually create a virus that can reduce virulence in its host. Furthermore, the likelihood of obtaining legal approval for the use of a genetically modified virus in Europe is for the moment very small. The conservation aspect is also a concern. Control of *H. annosum* is desirable in forestry, but at the same time we do not want to affect *H. annosum* in protected forests. A genetically modified virus released in nature cannot be controlled and the effects on the *H. annosum* population in protected forests could be severe.

References

Ahn, I.-P. & Lee, Y.-H. 2001. A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. *Molecular Plant-Microbe Interactions 14*, 496-507.

Anagnostakis, S.L. 1982. Biological control of chestnut blight. *Science 215*, 466-471.

Anagnostakis, S.L. 1987. Chestnut blight: the classical problem of an introduced pathogen. *Mycologia 79*, 23-27.

Anagnostakis, S.L. 1988. *Cryphonectria parasitica*, cause of chestnut blight. *Advances in Plant Pathology 6*, 123-136.

Anagnostakis, S.L., Chen, B., Geletka, L.M. & Nuss, D.L. 1998. Hypovirus transmission to ascospore progeny by field-released transgenic hypovirulent strains of *Cryphonectria parasitica*. *Phytopathology 88*, 598-604.

Anderson, N.A. 1984. Variation and heteokaryosis in *Rhizoctonia solani*. In: *The ecology and physiology of the fungal mycelium*. Ed. by Jennings, D.H. and Rayner, A.D.M. Cambridge University Press. Cambridge, UK. pp. 367-381.

Bandy, B.P. & Tavantzis, S.M. 1990. Effect of hypovirulent *Rhizoctonia solani* on rhizoctonia disease, growth, and development of potato plants. *American Potato Journal 67*, 189-200.

Barroso, G. & Labarere, J. 1997. Genetic evidence for nonrandom sorting of mitochondria in the basidiomycete *Agrocybe aegerita*. *Applied and Environmental Microbiology 63*, 4686-4691.

Barroso, G. & Labarere, J. 2000. Distribution and transmission of *Agrocybe aegerita* dsRNAs. In: *Science and cultivation of edible fungi, volume 1. Proceedings of the 15th International Congress on the Science and Cultivation of Edible Fungi, Maastricht, Netherlands, 2000*. Ed. by Van Griensven, L.J.L.D. Balkema. Rotterdam, The Netherlands. pp. 271-280.

Begueret, J., Turcq, B. & Clave, C. 1994. Vegetative incompatibility in filamentous fungi: *het* genes begin to talk. *Trends in Genetics 10*, 441-446.

Bendz-Hellgren, M., Lipponen, K., Solheim, H. & Thomsen, I.M. 1998. The Nordic countries. In: *Heterobasidion annosum: Biology, Ecology, Impact and Control*. Ed. by Woodward, S., Stenlid, J., Karjalainen, R. and Hüttermann, A. CAB International. Wallingford, UK. pp. 333-345.

Berbee, M.L. & Taylor, J.W. 1999. Fungal phylogeny. In: *Molecular fungal biology*. Ed. by Oliver, R. and Schweizer, M. Cambridge University Press. Cambridge, UK. pp. 21-77.

Bharathan, N. & Tavantzis, S.M. 1990. Genetic diversity of double-stranded RNA from *Rhizoctonia solani*. *Phytopathology 80*, 631-635.

Blattny, C. & Kralik, O. 1968. A virus disease of *Laccaria laccata* (Scop. ex Fr.) Cooke and some other fungi. *Ceska Mykologie 22*, 161-166.

Brandtberg, P.O., Johansson, M. & Seeger, P. 1996. Effects of season and urea treatment on infection of stumps of *Picea abies* by *Heterobasidion annosum* in stands on former arable land. *Scandinavian Journal of Forest Research 11*, 261-268.

Brasier, C.M. 1986. The d-factor in *Ceratocystis ulmi* - its biological characteristics and implications for Dutch elm disease. In: *Fungal virology*. Ed. by Buck, K.W. CRC Press. Boca Raton, FL. pp. 177-208.

Brasier, C.M. 1990. The unexpected element: mycovirus involvement in the outcome of two recent pandemics, Dutch elm disease and chestnut blight. In: *Pests, pathogens and plant communities*. Ed. by Burdon, J.J. and Leather, S.R. Blackwell Scientific Publications. Oxford, UK. pp. 289-307.

Brewer, B.J. & Fangman, W.L. 1980. Preferential inclusion of extrachromosomal genetic elements in yeast meiotic spores. *Proceedings of the National Academy of Sciences of the United States of America 77*, 5380-5384.

Brown, D.J.F., Robertson, W.M. & Trudgill, D.L. 1995. Transmission of viruses by plant nematodes. *Annual Review of Phytopathology 33*, 223-249.
Buck, K.W. 1979. Replication of double-stranded RNA mycoviruses. In: *Viruses and plasmids in fungi*. Ed. by Lemke, P.A. Marcel Dekker. New York, NY. pp. 93-160.
Buck, K.W. 1986. Fungal virology - an overview. In: *Fungal virology*. Ed. by Buck, K.W. CRC Press. Boca Raton, FL. pp. 1-84.
Buck, K.W. 1998. Molecular variability of viruses of fungi. In: *Molecular variability of fungal pathogens*. Ed. by Bridge, P., Couteaudier, Y. and Clarkson, J. CAB International. Wallingford, UK. pp. 53-72.
Campbell, R.N. 1996. Fungal transmission of plant viruses. *Annual Review of Phytopathology 34*, 87-108.
Capretti, P., Korhonen, K., Mugnai, L. & Romagnoli, C. 1990. An intersterility group of *Heterobasidion annosum* specialized to *Abies alba*. *European Journal of Forest Pathology 20*, 231-240.
Carpenter, C.E., Mueller, R.J., Kazmierczak, P., Zhang, L., Villalon, D.K. & Van Alfen, N.K. 1992. Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. *Molecular Plant-Microbe Interactions 5*, 55-61.
Castanho, B. & Butler, E.E. 1978. Rhizoctonia decline: a degenerative disease of *Rhizoctonia solani*. *Phytopathology 68*, 1505-1510.
Castanho, B., Butler, E.E. & Shepherd, R.J. 1978. The association of double-stranded RNA with Rhizoctonia decline. *Phytopathology 68*, 1515-1519.
Chase, T.E. & Ullrich, R.C. 1988. *Heterobasidion annosum*, root- and butt-rot of trees. *Advances in Plant Pathology 6*, 501-510.
Chen, B., Choi, G.H. & Nuss, D.L. 1993. Mitotic stability and nuclear inheritance of integrated viral cDNA in engineered hypovirulent strains of the chestnut blight fungus. *EMBO Journal 12*, 2991-2998.
Chen, B., Choi, G.H. & Nuss, D.L. 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science 264*, 1762-1764.
Choi, G.H. & Nuss, D.L. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science 257*, 800-803.
Chun, S.J. & Lee, Y.H. 1997. Inheritance of dsRNAs in the rice blast fungus, *Magnaporthe grisea*. *FEMS Microbiology Letters 148*, 159-162.
Coenen, A., Kevei, F. & Hoekstra, R.F. 1997. Factors affecting the spread of double-stranded RNA viruses in *Aspergillus nidulans*. *Genetical Research 69*, 1-10.
Day, P.R. & Anagnostakis, S.L. 1973. The killer system in *Ustilago maydis*: heterokaryon transfer and loss of determinants. *Phytopathology 63*, 1017-1018.
Day, P.R. & Dodds, J.A. 1979. Viruses of plant pathogenic fungi. In: *Viruses and plasmids in fungi*. Ed. by Lemke, P.A. Marcel Dekker. New York, NY. pp. 201-238.
Deacon, J.W. 1984. Introduction. *Introduction to modern mycology, second edition*. Blackwell Scientific Publications. Oxford, UK. pp 1-24.
Dickinson, M.J., Wellings, C.R. & Pryor, A. 1990. Variation in the double-stranded RNA phenotype between and within different rust species. *Canadian Journal of Botany 68*, 599-604.
Dieleman-Van Zaayen, A. 1970. Means by which virus disease in cultivated mushrooms is spread, and methods to prevent and control it. *Mushroom Growers Association Bulletin 244*, 158-178.
Dieleman-Van Zaayen, A. & Temmink, J.H.M. 1968. A virus disease of cultivated mushrooms in the Netherlands. *Netherland Journal of Plant Pathology 74*, 48-51.
El-Sherbeini, M. & Bostian, K.A. 1987. Viruses in fungi: Infection of yeast with the K1 and K2 killer viruses. *Proceedings of the National Academy of Sciences of the United States of America 84*, 4293-4297.

Elias, K.S. & Cotty, P.J. 1996. Incidence and stability of infection by double-stranded RNA genetic elements in *Aspergillus* section *flavi* and effects on aflatoxigenicity. *Canadian Journal of Botany 74*, 716-725.

Elliott, T.J. 1985. The general biology of the mushroom. In: *The biology and technology of the cultivated mushroom.* Ed. by Flegg, P.B., Spencer, D.M. and Wood, D.A. John Wiley & Sons. Chichester, UK. pp. 9-22.

Elliston, J.E. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. *Phytopathology 75*, 151-158.

Finkler, A., Koltin, Y., Barash, I., Sneh, B. & Pozniak, D. 1985. Isolation of a virus from virulent strains of *Rhizoctonia solani. Journal of General Virology 66*, 1221-1232.

Geels, F.P., Van de Geijn, J. & Rutjens, A.J. 1988. Pests and diseases. In: *The cultivation of mushrooms.* Ed. by Van Griensven, L.J.L.D. Balkema. Rotterdam, The Netherlands. pp. 361-422.

Ghabrial, S.A. 1980. Effects of fungal viruses on their hosts. *Annual Review of Phytopathology 18*, 441-461.

Ghabrial, S.A. 1986. A transmissible disease of *Helminthosporium victoriae* - Evidence for a viral etiology. In: *Fungal virology.* Ed. by Buck, K.W. CRC Press. Boca Raton, FL. pp. 163-176.

Ghabrial, S.A. 1994. New developments in fungal virology. *Advances in Virus Research 43*, 303-388.

Ghabrial, S.A. 1998. Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes 16*, 119-131.

Ghabrial, S.A. & Hillman, B.I. 1999. Partitiviruses-Fungal (*Partitiviridae*). In: *Encyclopedia of virology, second edition.* Ed. by Granoff, A. and Webster, R.G. Academic Press. San Diego, CA. pp. 1147-1151.

Griffiths, A.J.F. 1996. Mitochondrial inheritance in filamentous fungi. *Journal of Genetics 75*, 403-414.

Hansen, E.M., Stenlid, J. & Johansson, M. 1993. Genetic control of somatic incompatibility in the root-rotting basidiomycete *Heterobasidion annosum. Mycological Research 97*, 1229-1233.

Harrington, T.C., Worrall, J.J. & Rizzo, D.M. 1989. Compatibility among host-specialized isolates of *Heterobasidion annosum* from western North America. *Phytopathology 79*, 290-296.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. & Pegler, D.N. 1995. *Ainsworth & Bisby's Dictionary of the Fungi, 8th edition.* Cab International. Wallingford, UK.

Heiniger, U. & Rigling, D. 1994. Biological control of chestnut blight in Europe. *Annual Review of Phytopathology 32*, 581-599.

Hollings, M. 1962. Viruses associated with a die-back disease of cultivated muchroom. *Nature 196*, 962-965.

Hollings, M., Gandy, D.G. & Last, F.T. 1963. A virus disease of fungus: die-back of cultivated mushroom. *Endeavour 22*, 112-117.

Jian, J., Lakshman, D. & Tavantzis, S.M. 1997. Association of distinct double-stranded RNAs with enhanced or diminished virulence in *Rhizoctonia solani* infecting potato. *Molecular Plant-Microbe Interactions 10*, 1002-1009.

Khandjian, E.W., Turian, G. & Eisen, H. 1977. Characterization of the RNA mycovirus infecting *Allomyces arbuscula. Journal of General Virology 35*, 415-424.

Korhonen, K. 1978. Intersterility groups of *Heterobasidion annosum. Communicationes Instituti Forestalis Fenniae 94*, 1-25.

Korhonen, K., Delatour, C., Greig, B.J.W. & Schönhar, S. 1998. Silvicultural control. In: *Heterobasidion annosum: Biology, Ecology, Impact and Control.* Ed. by Woodward, S., Stenlid, J., Karjalainen, R. and Hüttermann, A. CAB International. Wallingford, UK. pp. 283-313.

Korhonen, K. & Stenlid, J. 1998. Biology of *Heterobasidion annosum*. In: *Heterobasidion annosum: Biology, Ecology, Impact and Control*. Ed. by Woodward, S., Stenlid, J., Karjalainen, R. and Hüttermann, A. CAB International. Wallingford, UK. pp. 43-70.

Lecoq, H., Boissonnet-Menès, M. & Delhotal, P. 1979. Infectivity and transmission of fungal viruses. In: *Fungal viruses*. Ed. by Molitoris, H.P., Hollings, M. and Wood, H.A. Springer-Verlag. Berlin, Germany. pp. 34-47.

Leslie, J.F. 1993. Fungal vegetative compatibility. *Annual Review of Phytopathology 31*, 127-150.

Lhoas, P. 1971. Infection of protoplasts from *Penicillium stoloniferum* with double-stranded RNA viruses. *Journal of General Virology 13*, 365-367.

Liu, Y.-C., Cortesi, P., Double, M.L., MacDonald, W.L. & Milgroom, M.G. 1996. Diversity and multilocus genetic structure in populations of *Cryphonectria parasitica*. *Phytopathology 86*, 1344-1351.

Liu, Y.-C. & Milgroom, M.G. 1996. Correlation between hypovirus transmission and the number of vegetative incompatibility (*vic*) genes different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology 86*, 79-86.

MacDonald, W.L. & Fulbright, D.W. 1991. Biological control of chestnut blight: use and limitations of transmissible hypovirulence. *Plant Disease 75*, 656-661.

Magliani, W., Conti, S., Gerloni, M., Bertolotti, D. & Polonelli, L. 1997. Yeast killer systems. *Clinical Microbiology Reviews 10*, 369-400.

Matsumoto, T. & Fukumasa, N. 1996. Mitochondrial DNA inheritance in sexual crosses of *Pleurotus ostreatus*. *Current Genetics 30*, 549-552.

May, G. & Taylor, J.W. 1988. Patterns of mating and mitochondrial DNA inheritance in the agaric basidiomycete *Coprinus cinereus*. *Genetics 118*, 213-220.

McFadden, J.J.P., Buck, K.W. & Rawlinson, C.J. 1983. Infrequent transmission of double-stranded RNA virus particles but absence of DNA proviruses in single ascospore cultures of *Gaeumannomyces graminis*. *Journal of General Virology 64*, 927-937.

Melzer, M.S. & Boland, G.J. 1996. Transmissible hypovirulence in *Sclerotinia minor*. *Canadian Journal of Plant Pathology 18*, 19-28.

Melzer, M.S., Dunn, M., Zhou, T. & Boland, G.J. 1997. Assessment of hypovirulent isolates of *Cryphonectria parasitica* for potential in biological control of chestnut blight. *Canadian Journal of Plant Pathology 19*, 69-77.

Milgroom, M.G. 1999. Viruses in fungal populations. In: *Structure and dynamics of fungal populations*. Ed. by Worrall, J.J. Kluwer Academic Publishers. Dordrecht, The Netherlands. pp. 283-306.

Niemelä, T. & Korhonen, K. 1998. Taxonomy of the genus *Heterobasidion*. In: *Heterobasidion annosum: Biology, Ecology, Impact and Control*. Ed. by Woodward, S., Stenlid, J., Karjalainen, R. and Hüttermann, A. CAB International. Wallingford, UK. pp. 27-33.

Nuss, D.L. & Koltin, Y. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. *Annual Review of Phytopathology 28*, 37-58.

Pallett, I.H. 1976. Interactions between fungi and their viruses. In: *Microbial and plant protoplasts*. Ed. by Peberdy, J.F., Rose, A.H., Rogers, H.J. and Cocking, E.C. Academic Press. London, UK. pp. 107-124.

Papp, T., Nyilasi, I., Fekete, C., Ferenczy, L. & Vagvolgyi, C. 2001. Presence of double-stranded RNA and virus-like particles in *Rhizopus* isolates. *Canadian Journal of Microbiology 47*, 443-447.

Peever, T.L., Liu, Y.C., Cortesi, P. & Milgroom, M.G. 2000. Variation in tolerance and virulence in the chestnut blight fungus-hypovirulence interaction. *Applied and Environmental Microbiology 66*, 4863-4869.

Perring, T.M., Gruenhagen, N.M. & Farrar, C.A. 1999. Management of plant viral diseases through chemical control of insect vectors. *Annual Review of Entomology 44*, 457-481.

Pfeiffer, I., Kucsera, J., Varga, J., Parducz, A. & Ferenczy, L. 1996. Variability and inheritance of double-stranded RNA viruses in *Phaffia rhodozyma*. *Current Genetics 30*, 294-297.

Polashock, J.J., Bedker, P.J. & Hillman, B.I. 1997. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: Ascospore inheritance and implications for mitochondrial recombination. *Molecular and General Genetics 256*, 566-571.

Pryor, A., Boelen, M.G., Dickinson, M.J. & Lawrence, G.J. 1990. Widespread incidence of double-stranded RNA of unknown function in rust fungi. *Canadian Journal of Botany 68*, 669-676.

Punja, Z.K. 1995. Influence of double-stranded RNAs on growth, sporulation, pathogenicity, and survival of *Chalara elegans*. *Canadian Journal of Botany 73*, 1001-1009.

Rayner, A.D.M., Coates, D., Ainsworth, A.M., Adams, T.J.H., Williams, E.N.D. & Todd, N.K. 1984. The biological consequences of the individualistic mycelium. In: *The ecology and physiology of the fungal mycelium*. Ed. by Jennings, D.H. and Rayner, A.D.M. Cambridge University Press. Cambridge, UK. pp. 509-540.

Rigling, D., Heiniger, U. & Hohl, H.R. 1989. Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. *Phytopathology 79*, 219-223.

Rogers, H.J., Buck, K.W. & Brasier, C.M. 1986. Transmission of double-stranded RNA and a disease factor in *Ophiostoma ulmi*. *Plant Pathology 35*, 277-287.

Rogers, H.J., Buck, K.W. & Brasier, C.M. 1987. A mitochondrial target for double-stranded RNA in diseased isolates of the fungus that causes Dutch elm disease. *Nature 329*, 558-560.

Romaine, C.P., Ulhrich, P. & Schlagnhaufer, B. 1993. Transmission of La France isometric virus during basidiosporogenesis in *Agaricus bisporus*. *Mycologia 85*, 175-179.

Roos, U.P., Khandjian, E.W. & Turian, G. 1976. RNA viruses in *Allomyces arbuscola*: Ultrastructural localization during the life-cycle. *Journal of General Microbiology 95*, 87-95.

Schisler, L.C., Sinden, J.W. & Sigel, E.M. 1963. Transmission of a virus disease of mushrooms by infected spores. *Phytopathology 53*, 888.

Schisler, L.C., Sinden, J.W. & Sigel, E.M. 1967. Etiology, symptomatology, and epidemiology of a virus disease of cultivated muchrooms. *Phytopathology 57*, 519-526.

Shen, X.R., Chen, M.J., Shen, J.Y., Pan, Y.J. & Gong, Z.X. 1993. The detection of single-stranded RNA in an isometric virus-like particle from Shiitake mushroom [*Lentinus edodes* (Berk.) Sing.]. *Annals of Applied Biology 122*, 271-277.

Sonnenberg, A.S.M. & Van Griensven, L.J.L.D. 1991. Evidence for transmission of La France disease in *Agaricus bisporus* by dsRNA. In: *Genetics and breeding of* Agaricus*: proceedings of the first international seminar on mushroom science, Mushroom Experimental Station, Horst, the Netherlands*. Ed. by Van Griensven, L.J.L.D. Pudoc. Wageningen, the Netherlands. pp. 109-113.

Sonnenberg, A.S.M., Van Kempen, I.P.J. & Van Griensven, L.J.L.D. 1995. Detection of *Agaricus bisporus* viral dsRNAs in pure cultures, spawn and spawn-run compost by RT-PCR. In: *Science and cultivation of edible fungi, volume 2: Proceedings of the 14th International Congress, Oxford,*. Ed. by Elliott, T.J. Balkema. Rotterdam, The Netherlands. pp. 587-594.

Stanway, C.A. & Buck, K.W. 1984. Infection of protoplasts of the wheat take-all fungus, *Gaeumannomyces graminis* var. *tritici*, with double-stranded RNA viruses. *Journal of General Virology 65*, 2061-2065.

Stenlid, J. & Wästerlund, I. 1986. Estimating the frequency of stem rot in *Picea abies* using an increment borer. *Scandinavian Journal of Forest Research 1*, 303-308.

Summerbell, R.C., Castle, A.J., Horgen, P.A. & Anderson, J.B. 1989. Inheritance of restriction fragment length polymorphisms in *Agaricus brunnescens*. *Genetics 123*, 293-300.

Sutherland, M.L. & Brasier, C.M. 1995. Effect of d-factors on in vitro cerato-ulmin production by the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. *Mycological Research 99*, 1211-1217.

Sutherland, M.L. & Brasier, C.M. 1997. A comparison of thirteen d-factors as potential biological control agents of *Ophiostoma novo-ulmi*. *Plant Pathology 46*, 680-693.

Swedjemark, G. & Stenlid, J. 1993. Population dynamics of the root-rot fungus *Heterobasidion annosum* following thinning of *Picea abies*. *OIKOS 66*, 247-254.

Tavantzis, S.M. 1994. Double-stranded RNA-associated cytoplasmic hypovirulence in *Rhizoctonia solani*: Prospects for developing a reliable, target-specific biocontrol system. In: *Advances in potato pest biology and management*. Ed. by Zehnder, G.W., Powelson, M.L., Jansson, R.K. and Raman, K.V. APS Press. St. Paul, MO. pp. 565-579.

Thor, M. & Stenlid, J. 1998. *Heterobasidion annosum* infection following mechanized first thinning and stump treatment in *Picea abies*. In: *Proceedings on Root and Butt Rots of Forest Trees, France 1997*. Ed. by Delatour, C., Guillaumin, J.J., Lung-Escarmant, B. and Marcais, B. INRA. Paris, France. pp. 397-407.

Vagvölgyi, C., Magyar, K., Papp, T., Vastag, M., Ferenczy, L., Hornok, L. & Fekete, C. 1998. Detection of double-stranded RNA molecules and virus-like particles in different *Mucor* species. *Antonie Van Leeuwenhoek 73*, 207-210.

Van Alfen, N.K., Hansen, D.R., Miller, S. & Barley, L. 1984. Cell-free transmission of hypovirulent phenotype of *Endothia parasitica*. *Phytopathology 74*, 833.

Van der Lende, T.R., Harmsen, M.C., Go, S.J. & Wessel, J.G.H. 1995. Double-stranded RNA mycoviruses in mycelium of *Pleurotus ostreatus*. *FEMS Microbiology Letters 125*, 51-56.

Van Diepeningen, A.D., Debets, A.J.M. & Hoekstra, R.F. 1998. Intra- and interspecies virus transfer in Aspergilli via protoplast fusion. *Fungal Genetics and Biology 25*, 171-180.

Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. & Wickner, R.B. 2000. *Virus Taxonomy - Classification and Nomenclature of viruses, Seventh report of the international committee on taxonomy of viruses*. Academic Press. London, UK.

Van Vuuren, H.J.J. & Jacobs, C.J. 1992. Killer yeasts in the wine industry: a review. *American Journal of Enology and Viticulture 43*, 119-128.

Van Zaayen, A. 1979. Mushroom viruses. In: *Viruses and plasmids in fungi*. Ed. by Lemke, P.A. Marcel Dekker. New York, NY. pp. 201-238.

Vannini, A., Smart, C.D. & Fulbright, D.W. 1993. The comparison of oxalic acid production in vivo and in vitro by virulent and hypovirulent *Cryphonectria (Endothia) parasitica*. *Physiological and Molecular Plant Pathology 43*, 443-451.

Varga, J., Kevei, F., Vagvolgyi, C., Vriesema, A. & Croft, J.H. 1994. Double-stranded RNA mycoviruses in section *Nigri* of the *Aspergillus* genus. *Canadian Journal of Microbiology 40*, 325-329.

Varga, J., Rinyu, E., Kevei, E., Toth, B. & Kozakiewicz, Z. 1998. Double-stranded RNA mycoviruses in species of *Aspergillus* sections *Circumdati* and *Fumigati*. *Canadian Journal of Microbiology 44*, 569-574.

Webber, J.F. 1993. D factors and their potential for controlling Dutch elm disease. In: *Dutch elm disease research*. Ed. by Sticklen, M.B. and Sherald, J.L. Springer-Verlag. New York, NY. pp. 322-332.

Worrall, J.J. 1997. Somatic incompatibility in basidiomycetes. *Mycologia 89*, 24-36.

Wu, J., Saupe, S.J. & Glass, N.L. 1998. Evidence for balancing selection operating at the *het*-c heterokaryon incompatibility locus in a group of filamentous fungi. *Proceedings of the National Academy of Sciences of the United States of America 95*, 12398-12403.

Zhang, R., Dickinson, M.J. & Pryor, A. 1994. Double-stranded RNAs in the rust fungi. *Annual Review of Phytopathology 32*, 115-133.
Zhou, T. & Boland, G.J. 1997. Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa. Phytopathology 87*, 147-153.
Zhou, T. & Boland, G.J. 1998. Suppression of dollar spot by hypovirulent isolates of *Sclerotinia homoeocarpa. Phytopathology 88*, 788-794.

Acknowledgements

A number of people have helped me during my studies and I am grateful to all of them.

I thank my supervisors Jan Stenlid and Elna Stenström, who actually believed in me and thought I could do this work!

I am grateful to Olov Pettersson, Juxian Zheng and Maria Jonsson for help in the lab, Åke Olson, Magnus Karlsson and Anna Rosling for ideas and discussions on the molecular work and all the other persons working in the PCR-lab for creating a pleasant atmosphere to work in.

I also thank Hanna Johannesson and Lena Jonsson for being such nice roommates, always prepared to give advice and help with all the minute little things life in a department consists of and to discuss techniques, fungi or anything else.

The MAaF group has contributed with interesting discussions about biocontrol and with nice company during trips and seminars.

I also want to thank everyone who has read my manuscripts, suggesting improvements and pointing out linguistic errors. You have all contributed to this thesis.

This work was financially supported by the Foundation for Strategic Environmental Research (MISTRA).