Biological Control of Grey Mould in Swedish Forest Nurseries

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Abstract

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Grey mould, caused by *Botrytis cinerea* Pers.:Fr., is a severe problem in conifer seedling production in Swedish forest nurseries. The overall aim of the study was to investigate the possibility of biological control and to find naturally occurring needle fungi with biosuppressive activity against grey mould on conifer seedlings in Swedish forest nurseries.

The potential of biological control was demonstrated by means of the microbial products Binab®, GlioMix® and Mycostop®. These products reduced *B. cinerea* infections in Scots pine seedlings by 16 to 57% in a field trial at a Swedish forest nursery, and by 51 to 94% in growth room assays under controlled climatic conditions. The effect of Binab® and GlioMix® was of the same level as the fungicide tolylfluanid.

A novel microcosm bioassay was developed to evaluate microorganisms for biocontrol of *B. cinerea* in co-inoculation tests on three-week-old Scots pine (*Pinus sylvestris* L.) seedlings. Pre-inoculation treatment by incubation at 40 °C for five hours predisposed seedlings to infection by *B. cinerea*. Different isolates of needle fungi were low to highly effective in suppression of *B. cinerea*. One of these isolates was identified as a *Fusarium* sp. strain F31 and was shown to secrete a spectrum of enniatins in liquid medium. Two new enniatins, J_2 and J_3, were co-isolated. Inhibitory activity to spore germination of *B. cinerea* was shown for the enniatins B, B_1, B_4 and K_1.

Based on the screening tests, nine fungal isolates were selected and evaluated for their efficacy to suppress spontaneous infections by *B. cinerea* in first-year Scots pine seedlings in a small-scale field experiment. Two isolates of *Trichoderma* sp. and the fungicide tolylfluanid showed some degree of control. The impact of the tested control agents on the community structure of needle-inhabiting fungi was analysed.

This thesis describes the first steps in the development of biological control of grey mould in forest nurseries. Further investigations are needed in the search for an efficient biocontrol method that can replace or complement chemical control.

Keywords: antibiotics, antifungal secondary metabolites, *Botryotinia fuckeliana*, depsipeptides, gray mold, scanning electron microscopy

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Appendix

Papers I-IV

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

I. Capieau K., Stenlid, J. & Stenström, E. 2004. Potential for biological control of *Botrytis cinerea* in *Pinus sylvestris* seedlings. *Scandinavian Journal of Forest Research*, *19*, 312-319.

II. Capieau K., Stenlid, J. & Stenström, E. Application of a novel bioassay to screen microorganisms for biocontrol of grey mould on Scots pine seedlings. (Manuscript).

III. Pohanka, A., Capieau, K., Broberg, A., Stenlid, J., Stenström, E. & Kenne, L. 2004. Enniatins of *Fusarium* sp. strain F31 and their inhibition of *Botrytis cinerea* spore germination. *Journal of Natural Products*, *67*, 851-857.

IV. Capieau K., Stenlid, J. & Stenström, E. The effect of antagonistic fungi and a fungicide on the incidence of *Botrytis cinerea* Pers.:Fr. and the community structure of needle-inhabiting fungi in *Pinus sylvestris* L. seedlings. (Manuscript).

Preface

This thesis is about grey mould. Most people are probably familiar with this fungal disease since it is extremely common, for example it attacks strawberries and grapes, converting them into grey, mouldy masses. To most people, these masses are a sign to trash their precious berries, for others they are worth further investigation and the look under a microscope, revealing a scenery of numerous grape-like clusters of oval spores occurring on interwoven, branched "threads". The spores are easily released and dispersed by wind to adjacent healthy berries or plant tissue, which in their turn may become infected.

Fungal diseases most probably have been known to mankind since the beginning of cultivation of agricultural crops. An ancient record on observation of fungal diseases originates from the important Roman researcher Gaius Plinius Secundus, generally known as Pliny the Elder (23-79 A.D.). He reported about rotting and decay of several varieties of grapes in his famous work "Naturalis Historia" (Book 14; chapter 4). Regarding grey mould on grapes, which is indeed mostly devastating for wine production (la pourriture grise), one has to mention that there exists a bright side as well: it can be beneficial in the production of some sweet, white, Botrytized dessert wines (*e.g.* Spätlese, Trockenbeerenauslese, Sauternes and Tokay) when infection of grapes results in "noble rot" (la pourriture noble), drying of the berries and concentration of the sugars.

Several years ago, I did my first experiments with grey mould during a plant pathology and crop protection course at the laboratory of Prof. John Mansfield in Wye (Kent, UK). Later, the subject of my MSc. thesis happened to be grey mould and in the laboratory of Prof. Monica Höfte in Gent (Belgium), we tried to unravel the mechanisms behind induced resistance to grey mould in bean plants upon inoculation of the roots with plant growth-promoting rhizobacteria. For some reason I arrived one year later in Uppsala and my quest for grey mould appeared to continue in conifer seedlings in Swedish forest nurseries.

Introduction

In Swedish forest nurseries, pine and spruce seedlings may become severely damaged by fungal diseases. Large monocultures of densely grown seedlings and high humidity within the seedling canopies often are conducive to fungal infection of the seedlings. Several factors may stress seedlings, which may become more susceptible to pathogenic attack. To prevent fungal problems and to reduce economic losses, forest nursery personnel have to rely on sanitation measures and the use of fungicides, which are the only control methods currently available. Of all fungal diseases occurring in Swedish forest nurseries, grey mould is the most common.

Background

Grey mould

Grey mould, caused by *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana* [de Bary] Whetzel), is an important fungal plant disease. The pathogen occurs worldwide, attacks at least 235 plant species and a wide range of different plant tissues (MacFarlane, 1968). The host range includes many economically important crops such as fruits and berries (grape, strawberry and raspberry), vegetables (tomato and cucumber), ornamentals (rose and gerbera), bulbs (onions) and forest tree seedlings (Jarvis, 1977). All broadleaf and conifer seedlings can be affected, but pines, spruces, *Abies* spp., hemlocks and Douglas-fir are very susceptible (Sutherland, 1991). Especially in intensive cropping systems, such as most of the greenhouse-grown and container-grown forest nursery seedlings, the risk for *B. cinerea* infections is high when the climatic conditions are favourable (Butin, 1995). In contrast, grey mould is rarely severe in bare-root seedlings produced in outdoor beds (Mittal *et al.*, 1987). Furthermore, *B. cinerea* may also infect and remain latent within plant tissues until conditions become favourable for further development of disease (Coley-Smith *et al.*, 1980). The process of infection by *B. cinerea* is often associated with rapid colonisation of wounded, predisposed, senescing or dead plant material as a nutrient-providing saprophytic base (Jarvis, 1977). *Botrytis cinerea* is as an unspecialised necrotroph that attacks above-ground portions of host plants, and that relies on a saprotrophic phase to complete its pathogenic life cycle (Bélanger & Avis, 2002)

Plant pathogens can be grouped according to their strategy for infection and survival. A minor part of fungal plant pathogens are biotrophs, which are obligate parasites that are generally relatively specialised in their host range and obtain their nutrition directly from living plant tissue (*e.g.* powdery mildews, rusts). The majority of plant pathogens are necrotrophs, killing plant tissues for their nutrition. *Botrytis cinerea* is a facultative parasite: it colonizes wounded, decaying or dead plant material, but it can also attack living plant tissue under certain conditions.

The fact that *B. cinerea* apparently does not display host specificity is in contrast with other species within the genus of *Botrytis*, for which the host range is considerably narrower (Jarvis, 1977). For example, *B. aclada* is found only on

Allium spp., *B. tulipae* on *Tulipa* sp. and *Lilium regale*, *B. fabae* on Leguminosae, and *B. pelargonii* on *Geranium* spp. (Jarvis, 1977).

Botrytis cinerea, species or complex?

Botrytis cinerea is a filamentous, haploid, heterothallic ascomycete exhibiting great morphological variability such as in mycelial growth rate, pathogenicity, incidence of sporulation structures and sclerotia, and fungicide resistance (*e.g.* Grindle, 1979; Lorbeer, 1980; Di Lenna *et al.*, 1981; Kerssies *et al.*, 1997). Several molecular-based studies on the genetic structure of field populations of *B. cinerea* showed a high degree of genetic diversity in *B. cinerea* and generally, no differentiation between the isolates could be attributed to parameters such as type of colonised plant tissue, geographic origin or sampling date (Van der Vlugt-Bergmans *et al.*, 1993; Giraud *et al.*, 1997; Kerssies *et al.*, 1997; Moyano *et al.*, 2003).

This phenotypic and genetic variability in *B. cinerea* has often been attributed to the multinucleate and heterokaryotic nature of conidia and hyphae, and to aneuploidy (Van der Vlugt-Bergmans *et al.*, 1993; Büttner *et al.*, 1994). Part of this variability might also be explained by sexual reproduction under natural conditions and by the activity of transposable elements (Giraud *et al.*, 1997; Giraud *et al.*, 1999). Giraud et al. (1997) found that populations of *B. cinerea* were divided into subpopulations, *transposa* and *vacuma*, that showed some degree of host specialization in grapes and other plant species in a vineyard in the Champagne region in France. This evidence contradicts the classical view of *B. cinerea* as a population with a high genetic diversity and without host specialisation (Giraud *et al.*, 1999). It remains to be shown whether host specificity exists in subpopulations of *B. cinerea* in other hosts such as forest nursery seedlings. An increased understanding of the population structure and dynamics, and the mode of reproduction in *B. cinerea* might be helpful in the development of efficient control measures.

Seedling production and fungal diseases in Swedish forest nurseries

The advent of container-grown production systems replacing bare root production systems, as has been the case in Swedish forest nurseries, resulted generally in the reduction of soilborne pathogens while the incidence of foliar diseases increased (Sutherland, 1991).

Seedling production

Greenhouse-grown container seedlings and field-grown bareroot stock are the two major types of forest tree nursery production systems with an annual global production of several billion seedlings (Colombo *et al.*, 2001). Swedish forest nurseries produce annually about 300 million seedlings of mainly Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* [L.] Karst.), which are necessary for the reforestation of about 132,000 ha annually (Skogsstatistisk årsbok, http://www.svo.se; in Swedish). Most of the seedlings in forest nurseries

are produced during one growing season for planting out on forest sites the following year. Sowing occurs from beginning of March until early summer with about 82% of all seedlings propagated in containers. Use of containerised systems leads to high seedling densities (*e.g.* Starpot® container-type with up to ca. 700 seedlings/m^2). After about two months in greenhouse facilities, seedling stock is moved to the forest nursery fields. Part of the Scots pine seedlings receives a long night treatment (16h darkness daily) for 2-3 weeks during May-June. This treatment induces the formation of double-needles on the new shoot, and thus these seedlings have the appearance of two-year-old seedlings obtained during one growing season (Dormling, 1986). Consequently, long-night treatment increase needle biomass, resulting in even more dense seedling canopies. The long-night treatment is also applied later in the growing season for both Scots pine (August) and Norway spruce (July), to hasten the winter hardening process in the seedlings (Dormling & Lundkvist, 1983). Container-grown seedlings in greenhouses and outdoor seedling beds are normally irrigated by overhead systems (Fig. 1) that can markedly prolong periods of high relative humidity and leaf wetness (Zhang & Sutton, 1994b). Leaf wetness periods of up to 120 hours were reported in canopies of Douglas-fir seedlings following overhead irrigation (Peterson *et al.*, 1988). At the end of the growing season (October-November), seedlings either remain in outdoor seedling beds, or are transported to large refrigerator or freezing rooms for overwinter storage.

Fig. 1. Container-grown conifer seedlings in outdoor seedling beds and greenhouses

Fungal diseases in Swedish forest nurseries

Most losses in conifer seeds and seedlings in forest nurseries are caused by mould fungi (Lawrence & Rediske, 1962; Mittal *et al.*, 1987). In Swedish forest nurseries, damage due to fungal diseases is by far the most severe problem. Large and dense monocultures with high humidity within seedling canopies favour the spread of fungal pathogens (Stenström & Arvidsson, 2001). Besides grey mould, other fungal diseases that may occur in Swedish forest nurseries are *Lophodermium* needle cast on pines (*Lophodermium seditiosum* [Minter, Staley & Millar]), pine twisting rust (*Melampsora pinitorqua* [de Bary] Rostrup), *Sirococcus* shoot blight on pine and spruce (*Sirococcus conigenus* [DC.] P.F. Cannon & Minter; formerly *Sirococcus strobilinus* Preuss), and Scleroderris canker (*Gremmeniella abietina* [Lagerberg] Morelet). Of these fungal diseases, grey mould was the most prevalent, according to a questionnaire that was sent to Swedish forest nurseries (Nyström *et al.*, 2001).

Predisposition of seedlings to disease

The tendency of non-genetic factors, acting prior to infection, to affect the susceptibility of plants to disease is called predisposition (Yarwood, 1959). Predisposition of host plants plays an important role in the biology of the infection process of *B. cinerea* (Jarvis, 1977). Stress factors include abnormal conditions of temperature, light or atmosphere, disturbance of water relationship, nutritional imbalance, toxic action of fungicides or other applied chemicals, or by injury from physical causes such as lightning and wind (Schoeneweiss, 1975,1981; Waller *et al.*, 2002). Predisposition of plants might be mediated either through physiological impairment, associated with reduced host defence reactions (Schoeneweiss, 1975), or through injury, permitting *B. cinerea* to enter and damage the plant (Jarvis, 1977).

Peterson et al. (1988) reported that low light intensities within dense canopies of conifer seedlings promoted needle senescence and subsequently increased incidence of *B. cinerea* in Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) seedlings. Zhang et al. (1995) concluded that light (400-700 nm wavelength) intensity of less than 10 μmol m^{-2} s^{-1} predisposed green needles of black spruce (*Picea mariana* [Mill.] B.S.P.) seedlings to infection by *B. cinerea*, and was associated with significant chlorophyll loss in the needles. Dead or dying needles in the lowermost portions of the seedlings can usually be found, and senescence of needles due to shading within dense canopies provides an additional substrate for colonisation by *B. cinerea*.

Other stress factors include drought stress (Zhang & Sutton, 1994b), abrupt chilling or freezing, long humid periods and poor nutrition state of seedlings (Mittal *et al.*, 1987). Furthermore, high temperature in combination with darkness predisposed black spruce seedlings to infection by *B. cinerea* (Zhang & Sutton, 1994b). The authors found that infection was successful in 2 to 6-month-old black spruce seedlings that were kept at 35 to 45 °C in darkness prior to inoculation with spores of *B. cinerea*, while grey mould did not develop in healthy unpredisposed seedlings. In late spring and summer, air temperature in greenhouses is often high (35-45 °C) and might thus lead to predisposition of seedlings to grey mould.

Infection by *Botrytis cinerea* and grey mould development

Mittal et al. (1987) reported that infection by *B. cinerea* might occur at all stages of seedling growth and seedling production: seeds, seedlings, during storage and transport to planting sites.

Seeds may be invaded by *B. cinerea*, resulting in seed decay and reduction of germination (Urosevic, 1961). Healthy, green tissue of seedlings might become infected directly, such as young shoots under humid conditions or very susceptible conifer species. More commonly, *B. cinerea* first becomes established on dead or moribund plant tissues that are usually present in the lowermost portions of the seedlings, and then spreads into adjacent healthy tissues or seedlings. Stem cankers originating from infected lower branches frequently girdle the stem and

kill the entire seedling (Mittal *et al.*, 1987). Symptoms include grayish-brown mould and conidiophores bearing hyaline or pale brownish conidia (Butin, 1995) and are frequently observed during late summer and early autumn after closure of seedling canopies (Sutherland, 1991). Development of grey mould during storage or transport is of particular importance since economic losses at these stages of seedling production are most pronounced. Seedling stock may be diseased, surface contaminated by propagules of *B. cinerea*, or carry latent infections originating in the field before entering the storage halls (Sutherland, 1991). Contamination of seedlings may also occur during packaging operations or during storage. The metabolism of *B. cinerea* is reduced at refrigerated temperatures (Jarvis, 1977). Low temperatures, however, do not kill the pathogen and when seedlings are returned to ambient conditions, grey mould may develop rapidly. A schematic overview of the disease cycle of grey mould is represented in Fig. 2.

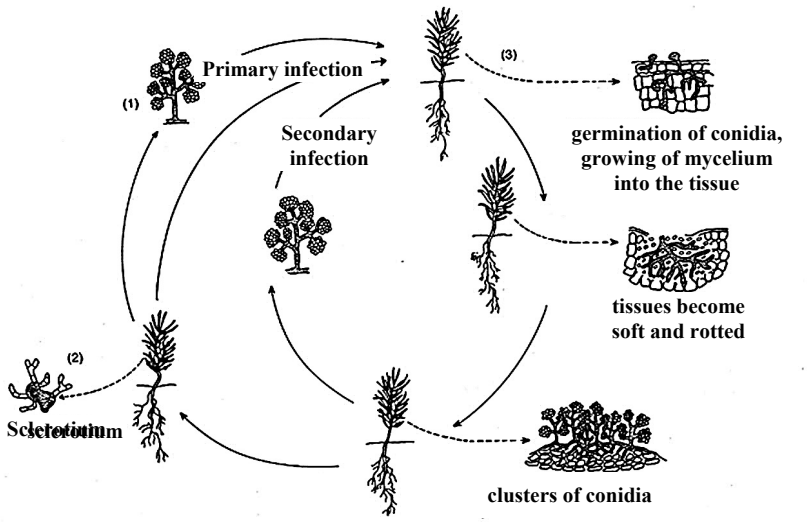


Fig. 2. Grey mould disease cycle; figure reproduced from Nef and Perrin (1999).

Sources of inoculum of *B. cinerea* include conidia, mycelial fragments and sclerotia, which may be disseminated to seedlings via wind, rain and irrigation water, insects, humans and culture practices. Conidia of *B. cinerea* can easily enter and escape greenhouses (Kerssies *et al.*, 1997) and rapidly colonise and contaminate forest nursery facilities. Germination and growth of the fungus following adhesion of spores to aerial plant surfaces depend on nutrients on the plant surface, temperature and leaf wetness (Blakeman, 1980; Elad, 1996). Conidia may germinate at temperatures ranging from zero to 26 °C (Jarvis, 1977). The most important factors that promote infection by *B. cinerea* are high humidity, free moisture on plant surfaces and cool weather conditions (Blakeman, 1980). Optimum temperatures for infection are between 10 °C and 20 °C, but grey mould may develop even at 2 °C (Jarvis, 1980; Elad *et al.*, 1989). Secretion of hydrolytic enzymes in advance of the growing hyphae, is crucial for infection by *B. cinerea*

(Verhoeff, 1980). Penetration of the host may be via wounds, stomata or directly through the host cuticle and occurs by means of infection pegs, simple and compound appressoria or infection cushions (Backhouse & Willets, 1987).

Current methods for control of grey mould in Swedish forest nurseries

Currently, cultural control and fungicidal applications are the two control measures employed in Swedish forest nurseries to control grey mould.

Cultural and sanitation measures, to reduce the risk of infection, include disinfestation of containers before sowing (*e.g.* Kohmann & Borja, 2002), use of almost pure growing substrate such as Sphagnum peat, reduction of sources of pathogen inoculum such as removal of diseased seedlings and crop debris, and increased spacing between seedlings. Increased seedling spacing to avoid predisposition of seedlings and to reduce humidity would be most helpful and was already recommended by Peace (1962). However, in current practice in Sweden, a reduction in seedling density would not be feasible.

Currently, the two chemical compounds that are allowed by the Swedish Chemical Inspectorate for control of grey mould in Swedish forest nurseries are tolylfluanid and iprodione. The latter is a dicarboximide fungicide that induces alterations to the germ tubes of *B. cinerea* and interferes with cell wall synthesis and cellular metabolism (Leroux, 1996). However, since the introduction of dicarboximides in the 1980's, several studies have reported on resistance in *B. cinerea* to dicarboximide fungicides (Katan, 1982; Wang *et al.*, 1986; van Steekelenburg, 1987; Elad *et al.*, 1992; Faretra & Pollastro, 1993a; Raposo *et al.*, 2000). Tolylfluanid is a fungicide belonging to another group of chemical compounds, phenylsulfamides, with a multisite mode of action (Chapeland *et al.*, 1999). Any major resistance problems to tolylfluanid have not been reported yet. However, resistance in field strains of *B. cinerea* has been shown for dichlofluanid, another phenylsulfamide compound (Pollastro *et al.*, 1996). Of the total amount of fungicides used in Swedish forest nurseries during 2000, tolylfluanid and iprodione accounted for about 62 and 21%, respectively (Hannerz & Nyström, 2001), enhancing the risk of a pathogen developing resistance to a particular overused fungicide (Stenström & Arvidsson, 2001)

There is a general increase in public awareness of the potential hazards posed by the use of pesticides, because of toxicological and environmental reasons. Reduced effectiveness of fungicides due to the emergence of resistance in fungal pathogens is maybe less well known to the public, but it is at least equally problematic. The history of chemical control of *B. cinerea* has shown that development of resistance is a recurring problem (Staub, 1991; Rosslenbroich & Stuebler, 2000). In the 1970's, field resistance to benzimidazoles (*e.g.* benomyl, carbendazim) and phenylcarbamates, two other groups of chemical compounds, was detected shortly after their introduction (Leroux, 1995). Occurrence of resistance in *B. cinerea* was also reported for more recently introduced chemical compounds such as anilinopyrimidines (Hilber & Schüepp, 1996; Leroux, 1996; Latorre *et al.*, 2002), phenylpyrroles (Faretra & Pollastro, 1993b) and also for the

hydroxyanilide fenhexamid (Baroffio *et al.*, 2003; Ziogas *et al.*, 2003). It is interesting to note that the majority of chemical compounds aim at a specific stage in the lifecycle of *B. cinerea*, inhibition of spore germination and/or germ tube elongation. In terms of resistance management, *B. cinerea* is a "high-risk pathogen" (Brent & Hollomon, 1998) and the emergence of resistant strains will continue to drive the search for novel fungicides, displaying different arrays of modes of action, and the implementation of anti-resistance management strategies (Rosslenbroich & Stuebler, 2000). Consequently, alternative measures to control grey mould are desirable and research aims at limiting the input of fungicides.

Biological control of *Botrytis cinerea*

Biological control can be defined as the reduction of the amount of inoculum or the disease producing activity of the pathogen accomplished through one or more organisms other than man (Cook & Baker, 1983). The ability of fungi to compete with, antagonise or actively parasitize other fungi, or to induce resistance mechanisms in host plants, has been exploited by humans to control pathogenic fungi. In the last decade, this has resulted in the development of several biocontrol products for usage in the agricultural and horticultural (Paulitz & Bélanger 2001). Products containing *Trichoderma harzianum*, *Ulocladium atrum* or *Bacillus subtilis* are used to control foliar pathogens such as *Botrytis* and powdery mildews (Paulitz & Bélanger 2001). No biological control products are registered for use in forest nurseries.

Grey mould can be suppressed by the introduction of filamentous fungi, yeasts or bacteria on the phylloplane of the host plant where these agents interfere with one or more key stages in the life cycle of the pathogen (Blakeman & Fokkema, 1982; Elad *et al.*, 1996). Two main strategies for biocontrol of *B. cinerea* are aimed at

-interference with the initial phases of infection by *B. cinerea*, *i.e.* microbial suppression directed at non-germinated or germinating spores of *B. cinerea*

-reduction of mycelial colonisation and survival during the saprotrophic stage of the life cycle of *B. cinerea* (Elad, 1996; Köhl & Fokkema, 1998).

Microorganisms introduced into the phyllosphere might affect germination of conidia of *B. cinerea* and subsequent host penetration through several mechanisms of which competition for nutrients and production of antifungal secondary metabolites have received most attention in research. Since germination of *B. cinerea* conidia is generally nutrient-dependent, microorganisms capable of efficient nutrient utilization should be good biocontrol agents of *B. cinerea* (Blakeman, 1993). Other possible mechanisms involved in interference with the infection process involve changed physical properties of the host surface, attachment of biocontrol agents to conidia of *B. cinerea*, production of substances interfering with the pathogenicity enzymes of *B. cinerea*, mycoparasitism, and induced resistance (Elad, 1996). The length of the time period during which an antagonist must interact with (germinating) conidia of *B. cinerea* on the leaf surface to prevent infection, is dependant on several factors influencing the

germination of conidia. Under favourable conditions, conidia may germinate within a few hours and in that case, antagonists need to possess relatively fast biocontrol mechanisms. Relatively fast-acting mechanisms involve competition for nutrients and antibiosis. Mechanisms that are slower in action involve induced resistance in the host plant, production of hydrolytic enzymes that degrade the cell walls of *B. cinerea* and parasitism (Köhl & Fokkema, 1998).

As needles senesce, nutrients become available that easily can be acquired by *B. cinerea*, as well as by other naturally occurring microorganisms. The strategy for biocontrol of *B. cinerea* in conifer seedlings could be aimed at prevention of colonisation by *B. cinerea* of dead and senescing needles in the lowermost parts of the seedlings (Peterson *et al.*, 1988). This would prevent the spread of developing mycelium of *B. cinerea* into adhering non-infected tissues and the build-up of spore inoculum that may cause secondary spread of the disease. Mechanisms behind this strategy of control of pathogen inoculum include colonisation of necrotic and senescent plant tissue by microorganisms, suppression of sporulation of *B. cinerea* and colonisation of sclerotia (Elad, 1996).

Aims of the study

The overall aim of the study was to investigate the possibility of finding naturally occurring needle fungi that have the ability to suppress grey mould on conifer seedlings in Swedish forest nurseries.

More specifically, the objectives were:

- to test the possibility of biological control, by means of commercially available microbial products, to suppress infection of Scots pine seedlings by *B. cinerea* under natural field conditions in Swedish forest nurseries.

- to develop a reliable screening method that can be used to evaluate microorganisms, isolated from needles of conifer seedlings originating from Swedish forest nurseries, for their potential for biocontrol of *B. cinerea* in Scots pine seedlings.

- to study antifungal secondary metabolites produced by a candidate biocontrol strain and to evaluate the biocontrol potential of this strain against *B. cinerea* on Scots pine seedlings under field conditions.

- to test the efficacy of a selected group of antagonistic fungi in suppressing infection of first-year Scots pine seedlings by *B. cinerea* in a small-scale field experiment.

- to characterise the impact of chemical and biological treatments on the fungal community in conifer needles

Results and discussion

Detection of *Botrytis*

Visual inspections of microscopic observation of tissues of conifer seedling for symptoms of grey mould are fast and may be very helpful in diagnosis. However, once symptoms of grey mould are obvious, control measures will not be very successful. For forest nursery personnel, it could thus be desirable to detect *B. cinerea* at early stages of infection. Useful and effective methods for detection of *B. cinerea* in infected, symptomless plant tissues include plating-out of tissues on agar media, immunological and molecular techniques.

The classical method of isolation of fungi involves placing small portions of plant tissue onto agar medium under aseptic conditions, followed by incubation and microscopic observation of characteristic fungal structures growing out from the tissues. This method was used for estimation of incidence of *B. cinerea* in needle segments in Paper **I** and **IV**. Addition of a herbicide to the water agar growth medium (**IV**), a method modified from Bannon (1978), provided the advantage of faster breakdown of green tissues and subsequently faster growth and sporulation of *B. cinerea*. Plating out is relatively cheap and provides useful information on the presence or absence of living fungal structures inside tissues, but the method has the disadvantage that it is highly time-consuming and that incidence of *B. cinerea* might be masked due to competition from other microorganisms growing out from the needle segments. This problem was encountered in Paper **IV**, and therefore other methods could be considered such as immunological-based techniques or molecular-based tools.

Molecular techniques based on the polymerase chain reaction (PCR) have been used as tool in the diagnosis of several fungal species. Dependent on the fungal species, different molecular-based approaches can be used to select suitable DNA sequences for design of specific primers, which can then be used to amplify a DNA sequence that is specific to a particular fungal species of *Botrytis*. For example, Rigotti *et al.* (2002) designed a 20-mer primer pair based on a 750-bp long randomly amplified DNA fragment that was present in all *B. cinerea* isolates obtained from several different hosts. Molecular techniques require relatively more expense and expertise. Furthermore, quantification of biomass of *Botrytis* in tissue samples is difficult due to the multinucleate nature of *Botrytis* conidia and hyphae, and problems associated with sampling from tissues and DNA extraction efficiency (Lévesque, 2001; Nielsen *et al.*, 2002; Walcott, 2003).

Immunological assays are based on the visualisation of the binding of a specific antibody to its respective antigen originating from a target organism. A commonly used assay is quantitative plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA). This is a laboratory-based, multistep assay in which antigens in plant extracts are trapped in the wells of a microtiter plate, in which the conversion of a substrate to a coloured product reports the presence of the target organism in the sample. Use of the highly *Botrytis* genus-specific monoclonal antibody BC-12.CA4, raised by Meyer and Dewey (2000), in PTA-ELISA, allowed the detection and quantification of *Botrytis* in pear stems (Meyer *et al.*,

2000), grape juice (Dewey *et al.*, 2000), tomatoes (Lurie *et al.*, 2003) and onion leaves (Yohalem *et al.*, 2004). Recently, a rapid (20 min.) and very user-friendly Tube immunoassay (TUBE-ELISA), modified from this PTA-ELISA, was developed to quantify the level of *Botrytis* rot in grapes on-site at wineries (Dewey & Meyer, 2004). The antibody BC-12.CA4 is highly stable and resistant to heat or freezing (Dewey, 2002a). Both DNA and PTA-ELISA-based detection methods have the disadvantage that forest nursery personnel cannot use them, since these methods require a well-equipped laboratory and expertise.

The antibody BC-12.CA4 has also been used in the development of a rapid (4 min) semi-quantitative *Botrytis* lateral flow device (B-LFD) (Frances M. Dewey, personal communication). The appearance of a "control" band after addition of a sample (plant extract diluted in a carrier buffer) reveals correct functioning of the device, and the appearance of a "test" band indicates that the sample contains *Botrytis*-specific antigens (Dewey, 2002b). This B-LFD was used to detect symptomless infections of *Botrytis* in artificially inoculated tomato fruit (Lurie *et al.*, 2003).

Table 1. *Botrytis Lateral Flow Tests with extracts from shoots of infected and symptomless Scots pine seedlings. Appearance of bands was observed 4, 30 and 90 min after inoculation of the devices*

Sample	Description sample[a]	Botrytis expected	C band 4 min	T band 4 min	T band 30 min	T band 90 min
1	Seedling heavily diseased with sporulation of *B. cinerea* visible	YES	YES	YES	YES	YES
2	As sample 1 + surface sterilisation[b]	YES	YES	YES	YES	YES
3	Symptomatic seedling but no sporulating *B. cinerea* visible	?	YES	NO	YES	YES
4	Sound-looking seedling	NO	YES	NO	NO	NO
5	Sound-looking seedling	NO	YES	NO	NO	YES

[a] Samples prepared by macerating the shoots in 1:20 (w/v) PBST buffer; PBST = phosphate buffered saline with 0.05% Tween 20

[b] 10s EtOH (70%), then 1min NaOCl (0.6%), followed by 3 rinses in sterile water

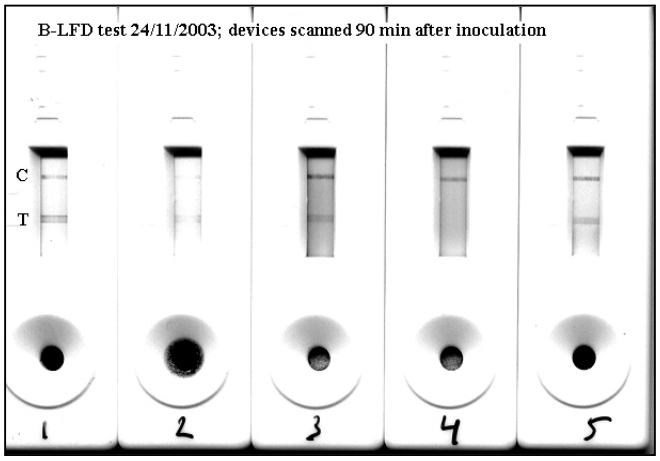


Fig. 3. *Botrytis*- Lateral Flow Devices for testing extracts from shoots of infected and symptomless Scots pine seedlings (see **Table 1** for a description of the samples).

The B-LFD (kindly provided by Frances M. Dewey) was also tested in preliminary experiments and found to be very helpful for detection of *Botrytis* in pine needles of 4-month-old Scots pine seedlings (Table 1 and Fig. 3) (Capieau et al., unpublished data). The intensity and the time needed for the appearance of the positive test band was proportional to the extent of grey mould development and thus to the amount of *Botrytis*-antigens present in the sample. The device is highly sensitive for detection of low amounts of antigen, is "user-friendly" and has potential to help forest nursery personal in the diagnosis of symptomless *Botrytis* infections.

Potential of biocontrol in Swedish forest nurseries (Paper I)

The possibility of biological control as an alternative to chemical control of grey mould was tested on Scots pine seedlings by means of three microbial products: the two biological control products Binab® TF.WP (Binab Bio-Innovation, Sweden) and Mycostop® (Verdera Oy, Finland), and the growth promoter product GlioMix® (Ticab AB, Sweden). The active agents in these three products are a mixture of *Trichoderma harzianum* and *T. polysporum*, the soilborne bacterium *Streptomyces griseoviridis* strain K61, and *Gliocladium* spp., respectively (**I**).

These products were initially tested for their biocontrol efficacy in growth room assays under controlled post-inoculation climatic conditions favouring the development of grey mould (>95% R.H., 20 °C). Prior to inoculation, the three-month-old Scots pine seedlings were predisposed to infection by the method described by Zhang & Sutton (1994b) for stressing of black spruce seedlings (incubation of seedlings in darkness at 35 °C for 4 days). This pre-inoculation treatment resulted in moderate to high infections of the needles and symptoms of grey mould development were frequently observed, when seedlings were inoculated with a suspension containing spores of *B. cinerea* only. It was thus concluded that the growth room assay was a suitable method for testing the efficacy of control agents (**I**). In the growth room assay, Mycostop, Binab and GlioMix reduced *B. cinerea* needle infections significantly by 51, 94 and 92%, respectively, compared to seedlings treated with a spore suspension of *B. cinerea* only. Furthermore, Binab and GlioMix suppressed *B. cinerea* as effectively as did the fungicide Euparen® (tolylfluanid).

Based on the results found in the growth room assays, the products were subsequently tested on Scots pine seedlings that were grown under routine practices in seedling beds at Swedish forest nurseries. In one successful field test, where a low to moderate incidence of natural grey mould was found in unprotected seedlings, repeated applications of treatments including Mycostop, Binab or GlioMix suppressed grey mould infections by 16 to 57% compared to the water control treatment and did not differ significantly from the fungicide treatments. However, only Mycostop (applied four times), and Binab (applied two times) were significantly different from the water control.

Interestingly, spraying a solution of 1% D-glucose in the field test reduced grey mould incidence by 64% and was significantly different from the water control treatment. One possible explanation might be that addition of glucose in the

present study stimulated a diverse phylloplane microflora. Certain microorganisms with the ability to acquire simple carbon sources might show antagonistic activity against *B. cinerea*. Such an effect has also been observed in other studies. An approximately 50% reduction in incidence of grey mould on strawberry fruits was found several times after the plants were treated with a 2% sucrose solution (John Sutton, personal communication). In another study, 2% sucrose in combination with 0.01% yeast extract in a water solution significantly reduced incidence of *B. cinerea* on the flowers of strawberry plants by 50%, as compared to the water control (McLean, 1988). Addition of a yeast isolate to the sucrose and yeast extract solution led to a further decrease in incidence of *B. cinerea* (McLean, 1988). However, the hypothesis that application of glucose solutions can stimulate naturally occurring antagonists on the phylloplane of conifer needles, resulting in reduced incidence of *B. cinerea*, requires much more evidence. In the small-scale field study of Paper **IV**, the glucose treatment did not suppress incidence of *B. cinerea* in needles of Scots pine seedlings. Incidence of *B. cinerea* in brown needles was not affected, while incidence increased significantly in yellow needles. There was a trend that the glucose treatment suppressed *B. cinerea* in green needles, comparable to the effect of the fungicide treatment, but incidence of *B. cinerea* in green needles was too low (< 5%) to draw final conclusions.

Development of bioassay and screening of isolates (Paper II)

A novel microcosm bioassay was developed to evaluate microorganisms for biocontrol of *B. cinerea* in co-inoculation tests on three-week-old predisposed Scots pine seedlings. This bioassay can be used along with other test systems such as the growth room assay (**I**), and *in vitro* interaction studies on agar media (**II**).

Effect of predisposition and first infection events

Zhang and Sutton (1994b) found that predisposition of black spruce seedlings (2-6 months old) to *B. cinerea* by the combination of pre-inoculation high temperature and darkness was quantitatively a function of the level and duration of treatment and seedling age. The design of the pre-inoculation treatment in our study (**II**) was based on these observations. In pilot studies, different pre-inoculation treatments of three-week-old Scots pine seedlings in darkness at high temperatures (30-40 °C) for different periods of time (0-10 h) were tested. The treatment of 40 °C for 5 h was found to be most suitable, since this treatment resulted in predisposition of the seedlings, but also because seedlings did not show symptoms of suffering injurious strain. Pre-inoculation treatments at 40 °C, lasting for more than 5 h often resulted in blight of the shoot and of the needle tips of the cotyledon needles.

Most of the conidia on the needle surface of unpredisposed seedlings failed to germinate at one and three days post inoculation (dpi). However, when seedlings were predisposed, germination of conidia and germ tube elongation were noticed already at one dpi, and hyphal growth and needle colonisation were obvious at three dpi. In several cases, hyphal tips grew toward epistomatal openings, but sometimes also appeared to avoid them. Penetration of needles was frequently observed to occur through epistomatal openings, and formation of appressoria was common (Fig. 4).

The predisposition of the seedlings by the treatment of 40 °C for 5 h may have involved exudation of solutes, many of which stimulate the germination and the growth of *B. cinerea* (Jarvis, 1977; Zhang & Sutton, 1994b). Whether this is a likely mechanism behind the predisposition effect remains doubtful. Electrolyte leakage from needles did not increase significantly when seedlings were treated for up to 5 h. A significant increase was detected when the high temperature treatment period was extended to 7 h. On the other hand, the very sensitive method of nuclear magnetic resonance (NMR) spectroscopy showed fluctuations in exudation of solutes from needles of predisposed three-week-old Scots pine seedlings (Capieau, K. and Pohanka, A., unpublished data). These NMR data showed no differences in the composition of the solutes between seedlings treated at 40 °C for 0 to 7 h, but there were minor differences in the amounts of solutes exuded between non-treated seedlings and seedlings treated for 3 to 7 h. Furthermore, transmission electron microscopy on sections of needles that were fixed immediately after the treatment of 40 °C for 5 h, showed plasmolysis in the needle epidermis cells (Fig. 5). This was due to increased evaporation rates and subsequent loss of cell turgor.

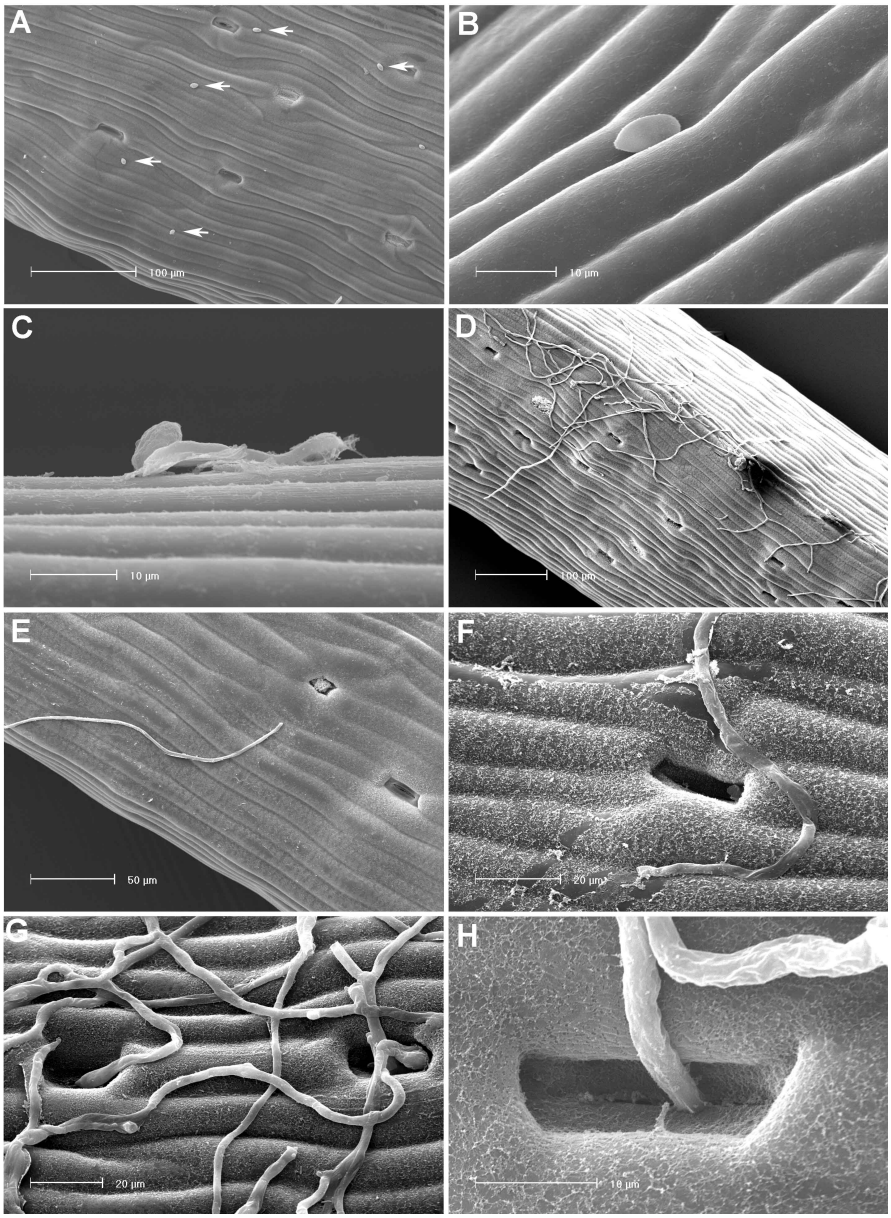


Fig. 4. Effect of predisposition of *Pinus sylvestris* seedlings on first infection events of needle tissues by *Botrytis cinerea*. Scanning electron micrographs of *B. cinerea* conidia on needles of unpredisposed Scots pine seedlings (A and B) at 3 days post inoculation (dpi), *B. cinerea* germ tube development at 1 dpi (C) and hyphal colonization of needle surface at 3 dpi (D) on predisposed seedlings, hyphal tips of *B. cinerea* showing taxis toward (E) and avoidance of stomatal openings (F), and penetration of stomata (G and H) on needles of predisposed seedlings.

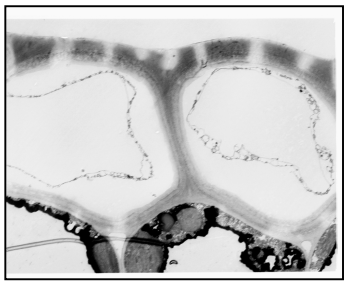


Fig. 5. Plasmolysis in epidermis cells of needles of three-week-old Scots pine seedlings. Needle sections were fixed for transmission electron microscopy immediately after the heat treatment of 40 °C for 5 h that predisposed seedlings to infection by *B. cinerea*.

Screening in the microcosm bioassay

Including the host plant during initial screening tests has the advantage over agar tests that it allows an examination of interactions between pathogen, biocontrol agent and the host. A number of important modes of action, which can not be tested in dual interaction tests on agar media, could be examined in the microcosm bioassay *e.g.* induced resistance, niche exclusion, competitive ability on the needle surface and needle colonizing ability. Furthermore, screening in this bioassay is relatively faster as compared to field tests (**I** and **IV**) or the growth room assay (Paper **I**) and allows examining the performance of biocontrol agents under specific environmental conditions.

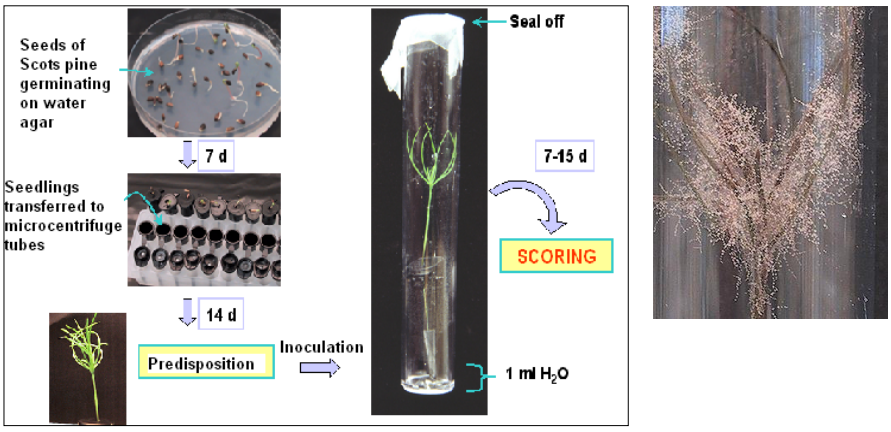


Fig. 6. Different steps involved in set-up of the microcosm bioassay that was used to screen microorganisms against *B. cinerea* on predisposed, young Scots pine seedlings. The right-hand picture shows grey mould in the bioassay, 10 days after inoculation of the needles with a suspension of *B. cinerea* spores (10^6 sp/mL).

A strong correlation was found for the relative effectiveness of the commercial products Mycostop, Binab and GlioMix and the fungicide tolylfluanid in suppressing *B. cinerea* between tests done in the microcosm bioassay and tests in the growth room assay and a field study performed in a Swedish forest nursery under natural conditions (**I**).

In total, 66 fungal and 11 bacterial isolates, three commercial microbial products and one fungicide (tolylfluanid) were evaluated for biocontrol of *B. cinerea* in the microcosm bioassay. Most of the filamentous fungal isolates suppressed *B. cinerea* to some degree (0-100%). Isolates of *Clonostachys rosea* (F02, F03 and F17) and *Gliocladium roseum* AFr710 were highly effective and reduced incidence of grey mould by 86 to 100%, while the product GlioMix reduced *B. cinerea* sporulation by 30%. In experiments on spruce seedlings in Canada, *G. roseum* AFr710 reduced grey mould as effectively as fungicides (Zhang & Sutton, 1994a). This strain prevented increased electrolyte leakage, loss of chlorophyll content and photosynthesis, normally associated with *B. cinerea* infection (Zhang *et al.*, 1996).

Isolates of *Trichoderma* spp. ranged from moderately to highly effective (13-75%) and the biocontrol product Binab TF.WP (*T. harzianum* and *T. polysporum*) reduced *B. cinerea* incidence by 19%. Of the other identified filamentous fungi, *Fusarium* sp. F31, *Cylindrocarpon* sp. F23, *Phoma* sp. F53, two isolates of *Lecythophora* sp. (F05 and F92) were moderately to highly effective. Isolates of *Penicillium* spp. were ineffective to only slightly effective (isolate F96). The yeast and bacterial isolates, as well as the biocontrol product Mycostop, were ineffective under the tested conditions and resulted, in some cases, in even increased incidences of *B. cinerea* sporulation (Fig. 3).

In conclusion, isolates of *Trichoderma* sp. and *Gliocladium* sp. were most effective at suppressing *B. cinerea* in the microcosm bioassay. The control efficacy of these isolates was comparable to that of the fungicide tolylfluanid and superior to the commercial microbial products.

Antifungal metabolites of *Fusarium* sp. F31 (Paper III)

Fusarium sp. strain F31 reduced incidence of *B. cinerea* in the microcosm bioassay by 84% (**II**). In co-operation with Pohanka et al. (Department of Chemistry, SLU), this strain was examined for production of antifungal secondary metabolites as one possible mechanism behind the observed suppressive effect. The *Fusarium* strain was cultured in liquid Hagem medium (Stenlid, 1985), and high performance liquid chromatography (HPLC) of the culture broth yielded several fractions inhibiting the spore germination of *B. cinerea* in a microtiter plate assay. Further purification and structure determination of the compounds comprised in these active fractions, revealed eight analogues of the compound enniatin. Two enniatins, J_2 and J_3, were isolated and characterised for the first time. Interestingly, these two were comprised in equal amounts within the same fraction (Fig. 7) and chromatographic resolution of the mixture was not successful. It was shown that J_2 and J_3 were isomers, which contained, in addition to three hydroxyisovaleric acid units, also the units N-methylated L-alanine, L-valine, and L-isoleucine, differing only in their primary sequence (Fig. 8.). The other enniatins isolated were known from previous studies.

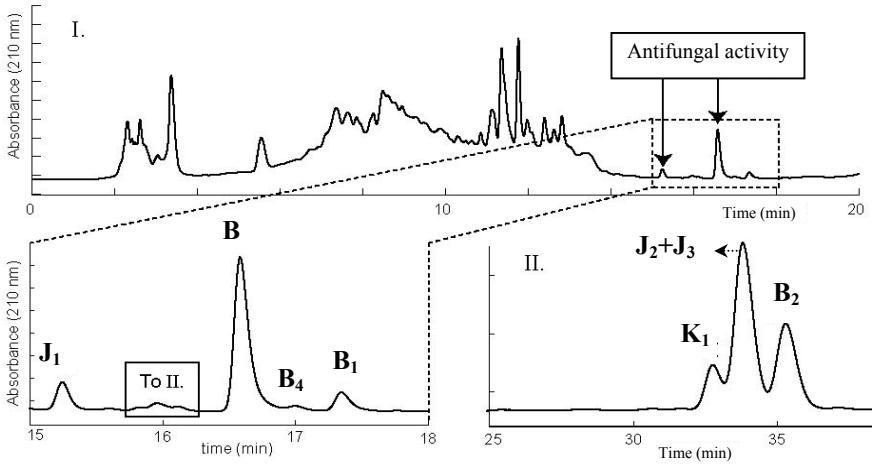
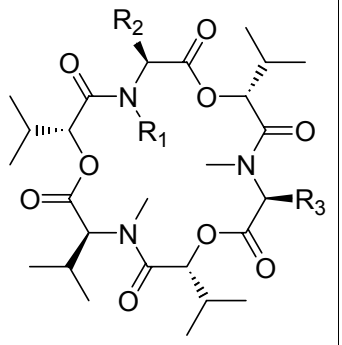


Fig. 7. Chromatographic separation of enniatins (bold letters) from *Fusarium* sp. strain F31. **I**: Gradient preparative, high performance liquid chromatography of 95% MeCN fraction resulting from solid phase extraction at 20-100% MeCN in H_2O in 10 min, with enniatin elution interval magnified at lower left. **II**: Isocratic preparative HPLC of boxed area at 63% MeCN in H_2O.

Four of the eight enniatins isolated from *Fusarium* sp. strain F31 were inhibitory to spore germination of *B. cinerea* (Fig. 8). Enniatin B_1 showed a partial inhibition of spore germination at a concentration of 25 µg/mL and full inhibition at 75 µg/mL. Partial inhibition of spore germination was found for enniatins B, B_4 and K_1.



Enniatin	R_1	R_2	R_3	Inhibition MIC[a]	Inhibition DI
J_1	Me	i-Pr	Me	>100	>100
B	Me	i-Pr	i-Pr	>100	100
B_4	Me	i-Pr	i-Bu	>100	50
B_1	Me	i-Pr	s-Bu	75	25
B_2	H	i-Pr	i-Pr	>100	>100
K_1	Me	i-Pr	Et	>100	100
J_2	Me	s-Bu	Me	-[b]	-
J_3	Me	Me	s-Bu	-	-

Fig. 8. Basic structure of enniatin and enniatin analogues isolated from *Fusarium* sp. strain F31 with data of minimum inhibitory concentration (MIC) and of detectable inhibitory concentration (DI) of enniatins (µg/mL) for spore inhibition of *Botrytis cinerea.*
[a]full inhibition of *B. cinerea* spore germination
[b]not determined

Several species of *Fusarium* produce enniatins that exhibit a wide range of biological properties such as phytotoxic (Burmeister & Plattner, 1987), antimicrobial (Tomoda *et al.*, 1992b), insecticidal activities (Grove & Pople, 1980) and inhibition of mammalian cholesterol acyltransferase activity (Tomoda *et al.*, 1992a). The well-established cationophoric properties of enniatins could be one mechanistic explanation of the broad spectrum of activity of this class of compounds (Shemyakin *et al.*, 1969).

Enniatins are low-molecular-weight, cyclic depsipeptides with a basic structure of three D-hydroxy acid residues and three L-amino acid residues alternately linked with ester and amide bonds forming an 18-membered ring (Fig. 8). The biosynthesis of enniatins is catalysed by the 347-kDa multifunctional enniatin synthetase (Zocher *et al.*, 1982). This polypeptide has all the functions necessary for synthesizing enniatins from their primary precursors D-2-hydroxyisovaleric acid and L-amino acids under consumption of ATP (Zocher *et al.*, 1982). The amino acid recognition site of enniatin synthetase does not show an absolute specificity and various strains of *Fusarium* show preferences for particular amino acids to be incorporated in the enniatin structure (Pieper *et al.*, 1992). For example, the enniatin synthetase from *Fusarium scirpi* Lambotte et Fautr. exhibits high affinity for valine, while the multienzyme of *Fusarium sambucinum* Fuckel shows specificity for leucine and isoleucine (Pieper *et al.*, 1992).

Extensive synthetic efforts as well as studies on directed biosynthesis by precursor feeding (*e.g.* addition of amino acids to liquid culture media) of enniatin producing *Fusarium* strains, have also been done to produce different enniatins (*e.g.* Krause *et al.*, 2001; Nilanonta *et al.*, 2003). In our study (**III**), enniatins J_1 and K_1 were isolated from the *Fusarium* sp. strain F31 for the first time without directed biosynthesis.

Although antibiosis might be one mode of action explaining the suppressive effect of *Fusarium* sp. strain F31 in the microcosm bioassay (**II**), it remains to be shown if production of enniatins also occurs *in planta*. Since most studies focus on the synthesis of enniatins, reports on the natural occurrence of these compounds are very scarce. The natural presence of enniatins was detected in a recent field study in Finland (Logrieco *et al.*, 2002) on wheat kernels infected predominantly by *Fusarium avenaceum* (Fr.) Sacc., the causal agent of head blight.

Performance testing of selected biocontrol isolates (Paper IV)

Spore suspensions of three isolates of *Trichoderma* sp. (F18, F58 and F99), two isolates of *Penicillium* sp. (F60 and F112), one of *Lecythophora* sp. (F05), *Clonostachys rosea* (F17), *Cylindrocarpon* sp. (F23) and *Fusarium* sp. (F31) were tested for their effect on incidence of *B. cinerea* in needles of first-year Scots pine (*Pinus sylvestris* L.) seedlings in a small-scale field study. The seedlings were treated during the growing season with four applications of the spore suspensions with approximately one month in between each spraying. Incidence of *B. cinerea* and other fungal species was checked two weeks after the third spraying and once more after the last spraying (October 2003). Overall incidence of *B. cinerea* was

low in unprotected seedlings of the water control treatment and decreased from about 18 to 9% between the two sampling periods. This low pathogen incidence complicated the assessment of the biocontrol potential of the tested isolates, since variability in results in suppression by the control agents is commonly observed in field studies.

At the first sampling time, the fungicide treatment (tolylfluanid) was most effective and reduced incidence of *B. cinerea* by 67%, while the biocontrol agents F18, F99 and F23 reduced incidence of *B. cinerea* significantly by 38, 39 and 40%, respectively, as compared to unprotected seedlings of the water control treatment. Statistically, the control efficacy of these three biocontrol agents was not different from the fungicide treatment. At the second sampling time, none of the treatments differed in control efficacy from the water control treatment.

A possible reason for the low efficacy could be explained by the relatively low abundance of the agents in the needles. Although populations of the biocontrol agents increased significantly in seedlings receiving the respective treatments (except for isolate *Lecytophora* sp. F05), the recovery rate of the tested agents was generally low, ranging from 3 to 17% out of the total needles sampled. The population size of the biocontrol agents might have been limited due to substrate competition from other naturally occurring needle fungi (see next section). Other factors that might have affected the colonisation of needles by the antagonists are water availability and temperature (Köhl & Fokkema, 1998).

Community of needle fungi in Scots pine seedlings (Paper IV)

The needle community structure was complex showing a high diversity of fungal species with only a few dominating ones. A high diversity and the presence of few dominating species is typical for the population of fungal species inhabiting different organs of a wide variety of plant species (Petrini *et al.*, 1992; Kowalski, 1993). Of the 78 isolated fungal species, only 14 fungal species showed a relatively high rate of incidence and of these, 4 species were present in more than 5% out of all investigated needles. *Botrytis cinerea*, *Phoma* sp., *Alternaria* sp. and *Phomopsis* sp., mainly associated with brown- and yellow-coloured needles, and *Cladosporium* sp., *Leptosphaeria* sp. and *Neofabreaea alba*, mainly colonising yellow and green needles, were among the fungal species that were isolated most frequently.

Brown- and yellow-coloured needles were more frequently colonised (80% and 63%, respectively) than were green-coloured needles (25%). Dying and dead needles are rapidly taken over by saprotrophs that have the ability to rapidly increase their population sizes on these substrates (Bélanger & Avis, 2002).

Competition from other naturally occurring needle fungi might be one possible reason for the observed low frequencies of *B. cinerea*. This might be evident from the addition of extra spore inoculum of *B. cinerea* to one field plot that did not increase infection rate by the pathogen. Production of antibiotics by microorganisms naturally occurring in the phyllosphere, can also interfere with the germination and the viability of conidia of *B. cinerea* (Elad, 1996).

The data of species composition was analysed by chi-square tests and detrended correspondence analysis (Fig. 9). The effects of type of treatment and needle colour accounted for most of the variation in the species composition. The fungicide treatment caused the largest shift, mainly due to a significant reduction in abundance of most of the fungal species, and was particularly evident at sampling time 2 where overall species richness was considerably less compared to fungal communities of the other treatments (**IV**). Interestingly, incidence of an unidentified yeast species was considerably higher in needles of fungicide treated seedlings, as compared to all other treatments. Certain phylloplane yeasts are resistant to a wide range of fungicides (Buck & Burpee, 2002) and are able to outcompete conidia of *B. cinerea* for nutrients when ratios of yeast cells to conidia of *B. cinerea* are high (50:1 to 100:1) (Helbig, 2001; Buck, 2002). It could thus be interesting to test the biocontrol potential of this yeast population into more detail.

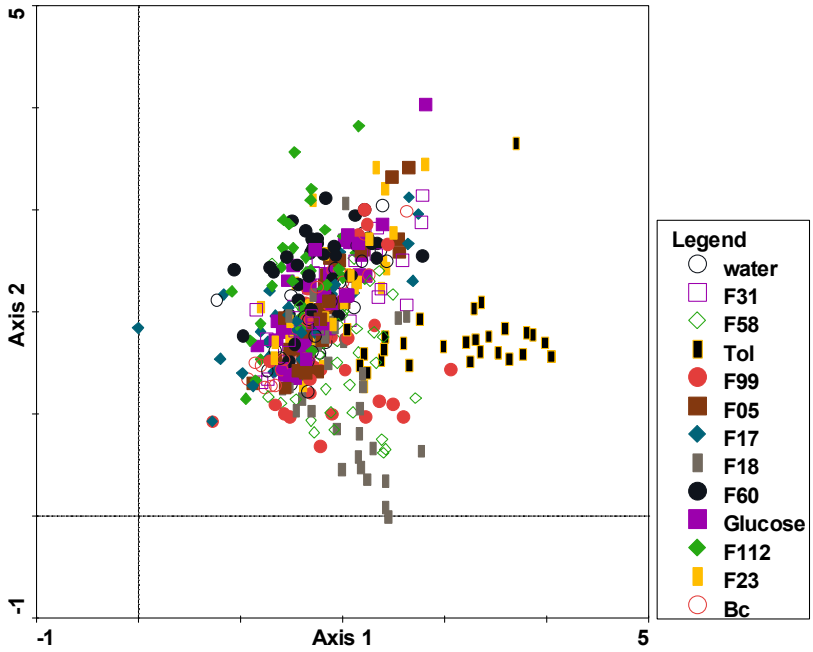


Fig. 9. Scatter of sample scores in the DCA1/DCA2 ordination space showing the distribution of samples according to treatment. The foliage of Scots pine seedlings was treated with 3 (sampling time 1) or 4 applications (sampling time 2) of water only (water control treatment), 2% D-Glucose, the fungicide tolylfluanid (Tol) or spore suspensions of *B. cinerea* (Bc), *Lecythophora* sp. F05, *Clonostachys rosea* F17, *Cylindrocarpon destructans* F23, *Fusarium* sp. F31, *Penicillium* sp. F60 or F112, or *Trichoderma* sp. F18, F58 or F99. Each sample includes the brown, yellow or green needle segments of one sampling unit.

Quantitatively, the fungicide treatment accounted for the largest impact on species composition inside the investigated needle segments, followed by the treatments of the three *Trichoderma* isolates and the two *Penicillium* isolates (Fig. 9). Qualitatively, no major effects of any of the biological control treatments on the resident fungal community were noticed (**IV**).

General discussion

Biological control has potential as an alternative control method to chemical control of *B. cinerea* causing grey mould disease on conifer seedlings in Swedish forest nurseries (**I**). This thesis describes the first steps in the development of a biocontrol method to suppress infections by *B. cinerea* in conifer seedlings in Swedish forest nurseries: isolation of microorganisms, development of screening methods to identify potential biocontrol candidates among isolates, revealing potential modes of actions and performance testing of selected biocontrol candidates under natural field conditions. However, more investigations are needed for the development of a biocontrol method that is highly efficient under field conditions.

Different test systems and assays were developed for the evaluation of biological control agents for their potential to control *B. cinerea* in pine seedlings. Depending on the purpose of the study, one or more of these systems might be appropriate to address certain research questions. The field tests performed at Swedish forest nurseries combined with the growth room assay were suitable for testing the effectiveness of commercial microbial products (**I**). Since these products are already available, one could primarily be interested in their potential to suppress grey mould in conifer seedlings under field conditions. The observed suppressive effect of the products Binab, GlioMix and Mycostop was recognised in one field experiment (**I**) and need to be confirmed, since the antagonistic effect might vary between different growing seasons and between different forest nurseries.

The growth room assay provided the advantage that a higher incidence of *B. cinerea* was obtained in the unprotected seedlings of the water control treatment (ca 51%), as compared to the forest nursery field test (ca 20%) (**I**). This was most probably due to stressing of the seedlings prior to inoculation and more favourable conditions for grey mould development in the growth room assay. It would be desirable to obtain higher infection levels in the forest nursery field test (**I**), as well in the small-scale field test (**IV**), to evaluate the full potential of biocontrol agents under natural conditions. Low levels of infection hardly allow detection of significant differences between treatments, since standard deviations from the treatments means are generally higher in field tests than in tests with controlled environmental conditions. Occasionally, there are reports from forest nurseries about serious and natural outbreaks of grey mould. It is thus obvious that further studies are required to investigate the factors that cause such outbreaks and to implement this knowledge in the design of biocontrol experiments with higher

levels of infection by *B. cinerea* in the water control treatment of field experiments.

Although screening of isolates of microorganisms against *B. cinerea* could be performed in field tests as well, such tests are laborious and require long experimental cycles. Initial screening programs should allow a relatively fast throughput of isolates resulting in the selection of potential candidate biocontrol agents. The microcosm bioassay (**II**) was found to be useful for this purpose. Screening of isolates was faster in this bioassay than what could be achieved in field tests or in the growth room assay of Paper **I**. Moreover, the host plant was included in this test system, which is considered advantageous over tests on agar media. Screening of isolates in this assay could be performed under a range of different temperatures and it also would be possible to test different combinations of varying concentrations of pathogen and biocontrol isolate in the inoculation suspension. In the microcosm bioassay, inoculum of pathogen and biocontrol agent were inoculated at the same time by dipping the foliage of the young, stressed Scots pine seedlings in the inoculation suspension. We tested the ability of the biocontrol isolates to interact with the conidia or with germinating conidia of *B. cinerea* on the needle phylloplane, thus interfering with the initial stages of infection. The ability of the tested isolates to suppress *B. cinerea* was judged from observation of incidence of sporulation of *B. cinerea* two weeks after inoculation. Although sporulation structures of *B. cinerea* might not have been visible in some co-inoculation tests, the pathogen might still have been able to infect and enter the needle tissues. For some of the most promising biocontrol isolates, it could thus be of interest to plate out surface sterilised needle tissues on agar media and check for incidence of *B. cinerea*. This could indicate whether the candidate biocontrol agents in the microcosm bioassay interfered with the germination of conidia of *B. cinerea* and prevented access to the infection court, or rather suppressed sporulation potential of the pathogen.

Most of the isolates that were screened in the microcosm bioassay (**II**) originated from conifer needles that were surface-washed, resulting in removal of most of the needle epiphytes, prior to isolation on water agar medium. It would be interesting to investigate the differences between populations of the needle epiphytic and endophytic microflora. The surprising effect of the glucose treatment in the forest nursery field study (**I**), gave rise to the hypothesis that the natural needle microflora that antagonise *B. cinerea* was stimulated. It is possible that such antagonists are epiphytes that easily can acquire these simple carbon sources. It could be of interest to investigate the population dynamics of needle epiphytes upon inoculation of glucose solutions. The small-scale field study (**IV)** did not show any impact of the glucose treatment on the community of needle fungi. In that study, needles were surface-washed prior to isolation of fungi and the obtained fungal community structures might thus not reflect the community structure of needle epiphytes. In contrast to the forest nursery field study (**I**), no suppressive effect on *B. cinerea* incidence by the glucose treatment was observed in the small-scale field study (**IV**). Either the hypothesis of the glucose effect is thus false, or no antagonists (or in too low abundances) were present in the needle microflora in the small-scale field study. If epiphytes are to be tested for their biocontrol potential against *B. cinerea*, it could thus be suggested to isolate

epiphytes from conifer needles of the same forest nursery environment as in the study of Paper **I**.

Only at sampling time 1 in the small-scale field study (**IV**), a significant suppressive effect against *B. cinerea* was noticed for the fungicide treatment and to a lower degree for three biocontrol isolates. No obvious field symptoms of grey mould could be observed throughout the experiment. The value of the small-scale field study regarding testing the control efficacy of the biocontrol isolates can thus be questioned. Incidence of *B. cinerea* inside needles was scored via plating out of surface-washed needle segments on agar medium and re-culturing of fungal colonies growing out from the segments. This scoring method (**IV**) was different from the one used in the microcosm bioassay (**II**). Since levels of *B. cinerea* were generally low inside the needles (**IV**), it is also possible that antagonism of *B. cinerea* on the phylloplane by the applied control agents or naturally occurring needle fungi might have been more substantial. Investigation of the epiphytic microflora in the small-scale field study could have reflected better the control efficacy results of the microcosm bioassay study. Population densities of the applied isolates might also have been higher on the needle phylloplane than inside the needles. Another factor accounting for the differences in control efficacy of the biocontrol isolates between the small-scale field study and the microcosm bioassay (**II**) might have been differences in environmental conditions. The young Scots pine seedlings in the microcosm bioassay (**II**) were also much more stressed prior to inoculation, as compared to the first-year, less susceptible seedlings in the field study (**IV**). Furthermore, interactions between *B. cinerea* and the applied biocontrol isolates might have been greatly influenced by needle microflora in the small-scale field study. In the microcosm bioassay, interactions between only the pathogen and the biocontrol agent on the needle phylloplane are observed, since no other microorganisms are added in this system.

It is possible that the observed *B. cinerea* infections were already established prior to the start of the small-scale field experiment (**IV**) and consequently little affected by surface application of antagonists. The first spraying was performed before seedling canopy closure, but brown and yellow needles were already present at that stage, allowing saprotrophic growth of *B. cinerea*. This might also partly explain why the fungicide treatment was not highly effective in the small-scale field experiment. Contact fungicides should be applied prior to establishment of the fungal pathogen in order to prevent infection. It may thus be necessary to apply control agents at even earlier stages of seedling production than the stage before canopy closure. Early application of effective antagonists could result in establishment of the antagonist prior to the natural needle microflora and prior to *B. cinerea*. It is important to perform the field experiments under such conditions as to test the control of natural infection courts of *B. cinerea*. Monitoring of *B. cinerea* field populations in conifer seedlings throughout the whole growing season could be of interest. Such studies may help in decision-making about timing of application of control agents.

The small-scale field study showed that *Fusarium* sp. F31 was not able to suppress naturally occurring grey mould in first-year Scots pine seedlings (**IV**), even if it was shown that *Fusarium* sp. F31 produces antifungal enniatins *in vitro*.

This might have been due to the observed low abundance levels of the *Fusarium* strain in needles following application of spore suspensions to the foliage of the seedlings and subsequent low concentrations of enniatin. However, levels of *Fusarium* might have been substantially higher in the epiphytic microflora, which could not be detected by the isolation method used in Paper **IV**. It would be interesting to perform tests on the direct effect of enniatin on *B. cinerea* and other needle fungi on the phylloplane. NMR studies could also provide information whether *Fusarium* sp. F31 can produce enniatins on the needle phylloplane. Such information would reveal whether antibiosis is a potential mechanism behind the antagonistic effect of *Fusarium* in the microcosm system.

The Botrytis Lateral Flow Device was tested because of two reasons. Firstly, the study in Paper **IV** revealed that alternative methods to plating-out of needle tissues on agar media for detection of *B. cinerea* could be desirable. Secondly, the device is simple to handle and it could help forest nursery personnel in diagnosis when infection by *B. cinerea* is suspected. However, detection of *B. cinerea* does not mean directly that application of fungicides is necessary. The device is very sensitive and the presence of hyphae of *B. cinerea* on the phylloplane of *e.g.* green needles, not necessarily resulting in infection, will also be detected. Most probably, tissues will have to be surface-washed or surface-sterilised, as was done in Papers **I** and **IV,** prior to processing of the sample in the B-LFD. More research is needed to find out how the B-LFD device can help in decision-making for application of control measures. However, detection of *B. cinerea* using the B-LFD device may indicate for forest nursery personnel to be observant. Monitoring of infected seedlings for grey mould development, minimising further stressing of seedlings and avoiding long humid periods could be advisable in these cases.

Future prospects

Through the course of this Ph.D. project, there has been an increasing awareness of the need for a better understanding of the infection process and epidemiology of *B. cinerea* in field conditions. Therefore, it could be of interest to investigate the structure and dynamics in populations of *B. cinerea* in conifer seedlings throughout the production season, including the storage period during winter, in Swedish forest nurseries. One possible tool to use could be population genetics to study the mode of reproduction (occurrence of sexual reproduction?) and the possible existence of subpopulations of *B. cinerea* in field conditions. Such information would be valuable in the development of efficient control methods and data of population dynamics could be helpful in deciding the best time to apply control measures.

Monitoring of the level of stress in seedlings might help in the development of prediction models for *B. cinerea* outbreaks. For example, it could be possible to test levels of stress by using the pine stilbene synthase cDNA tool (Schwekendiek *et al.*, 1992), which could help in determining the phases in seedling production that are most critical for attack by *B. cinerea*.

It has been demonstrated in other studies that combinations of several antagonists (*e.g.* Guetsky *et al.*, 2002), or applications of antagonists combined with fungicide sprayings in integrated pest management programs, can result in increased biocontrol efficacy than when each control agent is used alone. Such combinations could also be tested for biocontrol of grey mould in conifer seedlings for example by combining the most promising isolates from screening tests or by combinations of fungicides with *e.g.* the tolylfluanid tolerant yeast (**IV**).

A challenging field of research that remains to be explored is control of grey mould on seedlings during cold storage. Serious outbreaks of grey mould during cold storage have been observed previously in Swedish forest nurseries, leading to tremendous economic losses. It would be interesting to determine the factors that play a role in such outbreaks, as well as to test the possibility of biocontrol with cold-tolerant strains.

References

Backhouse, D. & Willets, H.J. 1987. Development and structure of infection cushions by *Botrytis cinerea*. *Transactions of the British Mycological Society 89*, 89-95.

Bannon, E. 1978. A method for detecting *Septoria nodorum* on symptomless leaves. *Irish Journal of Agricultural Research 17*, 323-325.

Baroffio, C.A., Siegfried, W. & Hilber, U.W. 2003. Long-term monitoring for resistance of *Botryotinia fuckeliana* to anilinopyrimidine, phenylpyrrole, and hydroxyanilide fungicides in Switzerland. *Plant Disease 87*, 662-666.

Bélanger, R.R. & Avis, T.J. 2002. Ecological processes and interactions occurring in leaf surface fungi. In: Lindow, S.E., Hecht-Poinar, E.I. &Elliot, V.J. (Eds.). *Phyllosphere microbiology*. The American Phytopathological Society, Minnesota, USA. pp.193-208

Blakeman, J.P. 1980. Behaviour of conidia on aerial plant surfaces. In: Coley-Smith, J.R., Verhoeff, K. &Jarvis, W.R. (Eds.). *The biology of Botrytis*. Academic Press Inc., London. pp.

Blakeman, J.P. 1993. Pathogens in the foliar environment. *Plant Pathology 42*, 479-493.

Blakeman, J.P. & Fokkema, N.J. 1982. Potential for biological control of plant diseases on the phylloplane. *Annual Review of Phytopathology 20*, 167-192.

Brent, K.J. & Hollomon, D.W. 1998. *Fungicide resistance: the assessment of risk. FRAC Monograph 2,* GCPF, Brussels, Belgium.

Buck, J. 2002. In vitro antagonism of *Botrytis cinerea* by phylloplane yeasts. *Canadian Journal of Botany 80*, 885-891.

Buck, J.W. & Burpee, L.L. 2002. The effects of fungicides on the phylloplane yeast populations of creeping bentgrass. *Canadian Journal of Microbiology 48*, 522-529.

Burmeister, H.R. & Plattner, R.D. 1987. Enniatin production by *Fusarium tricinctum* and its effects on germinating wheat seeds. *Phytopathology 77*, 1483-1487.

Butin, H. 1995. *Tree Diseases and Disorders*. Oxford University Press Inc., New York. 252 pp.

Büttner, P., Koch, F., Voigt, K., Quidde, T., Risch, S., Blaich, R., Bruckner, B. & Tudzynski, P. 1994. Variations in ploidy among isolates of *Botrytis cinerea*: implications for genetic and molecular analysis. *Current Genetics 25*, 445-450.

Chapeland, F., Fritz, R., Lanen, C., Gredt, M. & Leroux, P. 1999. Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea* (*Botryotinia fuckeliana*). *Pesticide Biochemistry and Physiology 64*, 85-100.

Coley-Smith, J.R., Verhoeff, K. & Jarvis, W.R. 1980. *The biology of Botrytis*. Academic Press Inc., London. 318 pp.

Colombo, S.J., Menzies, M.I. & O'Reilly, C. 2001. Influence of nursery cultural practices on cold hardiness of coniferous forest tree seedlings. In: Bigras, J. &Colombo, S.J. (Eds.). *Conifer cold hardiness*. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 223-252.

Cook, R.J. & Baker, K.F. 1983. *The nature an practice of biological control of plant pathogens*. American Phytopathological Society, St. Paul, Minnesota. 539 pp.

Dewey, F.M. 2002a. Botrytis antigens in wine. *The Australian and New Zealand Grapegrower and Winemaker, March issue.* pp. 20-21.

Dewey, F.M. 2002b. Immunological techniques. In: Waller, J.M., Lenné, J.M. &Waller, S.J. (Eds.). *Plant pathologist's pocketbook*. CABI Publishing, CAB International, Wallingford, Oxford, UK. pp. 221-228.

Dewey, F.M. & Meyer, U. 2004. Rapid, quantitative Tube immunoassays for on-site detection of *Botrytis*, *Aspergillus* and *Penicillium* antigens in grape juice. *Analytica Chimica Acta 513*, 11-19.

Dewey, F.M., S.E., E., Adams, D.O., Noble, A.C. & Meyer, U.M. 2000. Quantification of Botrytis in grape juice determined by a monoclonal antibody-based immunoassay. *American Journal of Viticulture and Enology 51*, 276-282.

Di Lenna, P., Marciano, P. & Magro, P. 1981. Comparative investigation on morphological and physiological features of three isolates of *Botrytis cinerea*. *Phytopathologische Zeitschrift 100*, 203-211.

Dormling, I. 1986. Dormancy in Scots pine (*Pinus sylvestris* L.) seedlings. In: Lindgren, D. (Ed.) *Provenances and forest tree breeding for high latitudes. Proceedings of the Frans Kempe symposium in Umeå, 10-11 June*. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Dormling, I. & Lundkvist, K. 1983. Vad bestämmer skogsplantors tillväxt och härdighet i plantskolan? *Skogsfakta, Nr 8. Sveriges Lantbruksuniversitet, Uppsala. (In Swedish.)*

Elad, Y. 1996. Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *European Journal of Plant Pathology 102*, 719-732.

Elad, Y., Malathrakis, M.E. & Dik, A.J. 1996. Biological control of *Botrytis*-incited diseases and powdery mildews in greenhouse crops. *Crop Protection 15*, 229-240.

Elad, Y., Yunis, H. & Katan, J. 1992. Multiple resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of *Botrytis cinerea* in Israel. *Plant Pathology 41*, 41-46.

Elad, Y., Yunis, H. & Mahrer, Y. 1989. Effect of climatic conditions in polyethylene-covered structures on grey mould disease of winter cucumber. *Applied Agricultural Research 3*, 243-247.

Faretra, F. & Pollastro, S. 1993a. Genetics of sexual compatibility and resistance to benzimidazole and dicarboximide fungicides in isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*) from nine countries). *Plant Pathology 42*, 48-57.

Faretra, F. & Pollastro, S. 1993b. Isolation, characterisation and genetic analysis of laboratory mutants of *Botryotinia fuckeliana* resistant to the phenylpyrrole fungicide CGA173506. *Mycological Research 97*, 620-624.

Giraud, T., Fortini, D., Levis, C., Lamarque, C., Leroux, P., LaBuglio, K. & Brygoo, Y. 1999. Two sibling species of the *Botrytis cinerea* complex, *transposa* and *vacuma*, are found in sympatry on numerous host plants. *Phytopathology 89*, 967-973.

Giraud, T., Fortini, D., Levis, C., Leroux, P. & Brygoo, Y. 1997. RFLP markers show genetic recombination in *Botryotinia fuckeliana* (*Botrytis cinerea*) and transposable elements reveal two sympatric species. *Molecular Biology and Evolution 14*, 1177-1185.

Grindle, M. 1979. Phenotypic differences between natural and induced variants of *Botrytis cinerea*. *Journal of General Microbiology 111*, 109-120.

Grove, J.F. & Pople, M. 1980. The insecticidal activity of beauvericin and the enniatin complex. *Mycopathologia 70*, 103-105.

Guetsky, R., Shtienberg, D., Elad, Y., Fisher, E. & Dinoor, A. 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology 92*, 976-985.

Hannerz, M. & Nyström, C. 2001. Ingen minskad användning av bekämpningsmedel. *Plantaktuellt Nr 4, SkogForsk, Uppsala, Sweden. 3pp. (In Swedish)*.

Helbig, J. 2001. Field and laboratory investigations into the effectiveness of *Rhodotorula glutinis* (isolate 10391) against *Botrytis cinerea* Pers. ex Fr. in strawberry. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 108*, 356-368.

Hilber, U.W. & Schüepp 1996. A reliable method for testing the sensitivity of *Botryotinia fuckeliana* to anilinopyrimidines in vitro. *Pesticide Science 47*, 241-247.

Jarvis, W.R. 1977. *Botryotinia and Botrytis Species: Taxonomy, Physiology, and Pathogenicity*. Hignell Printing Limited, Research Branch, Department of Agriculture, Ottawa, Canada. 195 pp.

Jarvis, W.R. 1980. Epidemiology. In: Coley-Smith, J.R., Verhoeff, K. &Jarvis, W.R. (Eds.). *The biology of Botrytis*. Academic Press Inc., London. pp. 219-250.

Katan, T. 1982. Resistance to 3,5-dichlorophenyl-*N*-cyclimide (dicarboximide) fungicides in the grey mould pathogen *Botrytis cinerea* in protected crops. *Plant Pathology 31*, 133-141.

Kerssies, A., Bosker-van Zessen, A.I., Wagemakers, C.A.M. & van Kan, J.A.L. 1997. Variation in Pathogenicity and DNA polymorphism among *Botrytis cinerea* insolates sampled inside and outside a glasshouse. *Plant Disease 81*, 781-786.

Kohmann, K. & Borja, I. 2002. Hot-water treatment for sanitizing forest nursery containers: Effects on container microflora and seedling growth. *Scandinavian Journal of Forest Research 17*, 111-117.

Kowalski, T. 1993. Fungi living in symptomless needles of *Pinus sylvestris* with respect to some observed disease processes. *Journal of Phytopathology 139*, 129-145.

Krause, M., Lindemann, A., Glinski, M., Hornbogen, T., Bonse, G., Jeschke, P., Thielking, G., Gau, W., Kleinkauf, H. & Zocher, R. 2001. Directed biosynthesis of new enniatins. *Journal of Antibiotics 54*, 797-804.

Köhl, J. & Fokkema, N.J. 1998. Strategies for biological control of necrotrophic fungal foliar pathogens. In: Boland, G.J. &Kuykendall, L.D. (Eds.). *Plant-microbe interactions and biological control*. Marcel Dekker, Inc., New York, USA. pp 49-88.

Latorre, B.A., Spadaro, I. & Rioja, M.E. 2002. Occurence of resistant strains of Botrytis cinerea to anilinopyrimidine fungicides in table grapes in Chile. *Crop Protection 21*, 957-961.

Lawrence, W.H. & Rediske, J.H. 1962. Fate of sown Douglas-fir seed. *Forest Science 8*, 210-218.

Leroux, P. 1995. Progress and problems in the control of *Botrytis cinerea* in grapevine. *Pesticide Outlook 6*, 13-19.

Leroux, P. 1996. Recent developments in the mode of action of fungicides. *Pesticide Science 47*, 191-197.

Lévesque, C.A. 2001. Molecular methods for detection of plant pathogens. *Candian Journal of Plant Pathology 24*, 333-336.

Logrieco, A., Rizzo, A., Ferracane, R. & Ritieni, A. 2002. Occurence of beauvericin and enniatins in wheat affected by *Fusarium avenaceum* head blight. *Applied and Environmental Microbiology, 68,* 82-85.

Lorbeer, J.W. 1980. Variation in *Botrytis* and *Botryotinia*. In: Coley-Smith, J.R., Verhoeff, K. &Jarvis, W.R. (Eds.). *The biology of Botrytis*. Academic Press Inc. (London) LTD. Pp 19-40.

Lurie, S., Powell, A.L.T., Dewey, F.M., Martin, R., Labavitch, J.M. & Bennett, A.B. 2003. Endogenous expression of cell wall enzymes in tomato fruit affects decay development. *XIth International Congress on Molecular Plant-Microbe Interactions, St Petersburg, Russia, July 18-27* Abstracts, p. 256.

MacFarlane, H.H. 1968. Plant host-pathogen index to volumes 1-40 (1922-1961) od Review of Applied Mycology. Commonwealth Mycological Institute, Kew, England.

McLean, M.A. 1988. *The microflora of strawberry in relation to biological control of grey mould fruit rot caused by Botrytis cinerea Pers.:Fr. MSc. Thesis, University of Guelph, Canada*.

Meyer, U. & Dewey, F.M. 2000. Efficacy of different immunogens for raising monoclonal antibodies to *Botrytis cinerea*. *Mycological Research 104*, 979-987.

Meyer, U., Spotts, R.A. & Dewey, F.M. 2000. Immunological detection and quantification of *Botrytis cinerea* in pear stems during cold storage. *Plant Disease 84*, 1099-1103.

Mittal, R.K., Singh, P. & Wang, B.S.P. 1987. *Botrytis*: a hazard to reforestation. *European Journal of Plant Pathology 17*, 369-384.
Moyano, C., Alfonso, C., Gallego, J., Raposo, R. & Malgarejo, P. 2003. Comparison of RAPD and AFLP marker analysis as a means study the genetic structure of *Botrytis cinerea* populations. *European Journal of Plant Pathology 109*, 515-522.
Nef, L. & Perrin, R. 1999. *Practical handbook on damaging agents in the European forest nurseries*. European Union, AIR 2-CT93-1694 Project, 352 pp.
Nielsen, K., Yohalem, D.S. & Jensen, D.F. 2002. PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion. *Plant Disease 86*, 682-686.
Nilanonta, C., Isaka, M., Chanphen, R., Thong-orn, N., Tanticharoen, M. & Thebtaranonth, Y. 2003. Unusual enniatins produced by the insect pathogenic fungus *Verticillium hemipterigenum*: isolation and studies on precursor-directed biosynthesis. *Tetrahedron 59*, 1015-1020.
Nyström, C., Hannerz, M., Stenström, E. & Lindelöw, Å. 2001. Enkätundersökning om skogsplantskolornas miljöpåverkan. SkogForsk, Uppsala, Sweden. Arbetsrapport Nr 484, 13 pp. ISSN 1404-305X.
Peace, T.R. 1962. *Pathology of trees and shrubs with special reference to Britain*. Clarendon Press, Oxford. 753 pp.
Peterson, M.J., Sutherland, J.R. & Tuller, S.E. 1988. Greenhouse environment and epidemiology of grey mould of container-grown Douglas-fir seedlings. *Canadian Journal of Forest Research 18*, 974-980.
Petrini, O., Sieber, T.N., Toti, L. & Viret, O. 1992. Ecology, metabolite production, and substrate utilization in endophytic fungi. *Natural toxins 1*, 185-196.
Pieper, R., Kleinkauf, H. & Zocher, R. 1992. Enniatin synthetases from different Fusaria exhibiting distinct amino acid specificities. *Journal of Antibiotics 45*, 1273-1277.
Pollastro, S., Faretra, F., Di Canio, V. & De Guido, A. 1996. Characterization and genetic analysis of field isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*) resistant to dichlofluanid. *European Journal of Plant Pathology 102*, 607-613.
Raposo, R., Gomez, V., Urrutia, T. & Melgarejo, P. 2000. Fitness of *Botrytis cinerea* associated with dicarboximide resistance. *Phytopathology 90*, 1246-1249.
Rigotti, S., Gindro, K., Richter, H. & Viret, O. 2002. Characterization of molecular markers for specific and sensitive detection of Botrytis cinerea Pers.:Fr. in strawberry (Fragaria x ananassa Duch.) using PCR. *FEMS Microbiology Letters 209*, 169-174.
Rosslenbroich, H.J. & Stuebler, D. 2000. Botrytis cinerea - history of chemical control and novel fungicides for its management. *Crop Protection 19*, 557-561.
Schoeneweiss, D.F. 1975. Predisposition, stress, and plant disease. *Annual Review of Phytopathology 13*, 193-211.
Schoeneweiss, D.F. 1981. The role of environmental stress in diseases of woody plants. *Plant Disease 65*, 308-314.
Schwekendiek, A., Pfeffer, G. & Kindl, H. 1992. Pine stilbene synthase cDNA, a tool for probing environmental stress. *FEBS Letters 301*, 41-44.
Shemyakin, M.M., Ovchinnikov, Y.A., Ivanov, N.T., Antonov, V.K., Vinogradova, E.I., Shkrob, A.M., Malenkov, G.G., Evstratov, A.V., Laine, I.A., Melnik, E.I. & Ryabova, I.D. 1969. Cyclodepsipeptides as chemical tools for studying ionic transport through membranes. *Journal of Membrane Biology 1*, 402-430.
Staub, T. 1991. Fungicide resistance: practical experience with antiresistance strategies and role of integrated use. *Annual Review of Phytopathology 29*, 421-442.
Stenlid, J. 1985. Population structure of *Heterobasidion annosum* as determined by somatic incompatibility , sexual incompatibility and isozyme patterns. *Canadian Journal of Botany 63*, 2268-2273.
Stenström, E. & Arvidsson, B. 2001. Fungicidal control of *Lophodermium seditiosum* on *Pinus sylvestris* seedlings in Swedish forest nurseries. *Scandinavian Journal of Forest Research 16*, 147-154.
Sutherland, J.R. 1991. Management of pathogens in seed orchards and nurseries. *The Forestry Chronicle 67*, 481-485.

Tomoda, H., Huang, X.H., Cao, J., Nishida, H., Nagoa, R., Okuda, S., Tanaka, H. & Omura, S. 1992a. Inhibition of acyl-CoA: cholesterol acyltransferase activity by cyclodepsipeptide antibiotics. *Journal of Antibiotics 45*, 1626-1632.

Tomoda, H., Nishida, H., Huang, X.H., Masuma, R., Kim, Y.K. & Omura, S. 1992b. New cyclodepsipeptides, enniatins D, E and F produced by *Fusarium* sp. FO-1305. *Journal of Antibiotics 45*, 2167-2170.

Urosevic, B. 1961. The influence of saprophytic and semisaprophytic fungi on the germination of Norway spruce and Scots pine seeds. *Proceedings of the International Seed Testing Association 26*, 537-556.

Walcott, R. 2003. Detection of seedborne pathogens. *HortTechnology 13*, 40-47.

Waller, J.M., Lenné, J.M. & Waller, S.J. 2002. *Plant pathologist's pocketbook*. CABI Publishing, CAB International, Wallingford, UK. 516 pp.

Van der Vlugt-Bergmans, C.J.B., Brandwagt, B.F., Van 't Klooster, J.W., Wagemakers, C.A.M. & van Kan, J.A.L. 1993. Genetic variation and segregation of DNA polymorphisms in *Botrytis cinerea*. *Mycological Research 97*, 1193-1200.

van Steekelenburg, N.A.M. 1987. Resistance to benzimidazole and dicarboximide fungicides in *Botrytis cinerea* and *Dydimella bryoniae* in cucumbers in the Netherlands. *Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent 52*, 875-880.

Wang, Z.-N., Coley-Smith, J.R. & Wareing, P.W. 1986. Dicarboximide resistance in *Botrytis cinerea* in protected lettuce. *Plant Pathology 35*, 427-433.

Verhoeff, K. 1980. The infection process and host-pathogen interactions. In: Coley-Smith, J.R., Verhoeff, K. &Jarvis, W.R. (Eds.). *The biology of Botrytis*. Academic Press Inc., London. pp 153-180.

Yarwood, C.E. 1959. Predisposition. In: Horsfall, J.G. &Dimond, A.E. (Eds.). *Plant Pathology*. New York & London: Academic. pp. 521-562.

Yohalem, D.S., Nielsen, K., Green, H. & Jensen, D.F. 2004. Biocontrol agents efficiently inhibit sporulation of Botrytis aclada on necrotic leaf tips but spread to adjacent living tissue is not prevented. *FEMS Microbiology and Ecology 47*, 297-303.

Zhang, P.G. & Sutton, J.C. 1994a. Evaluation of microorganisms for biocontrol of *Botrytis cinerea* in container-grown black spruce seedlings. *Canadian Journal of Forest Research 24*, 1312-1316.

Zhang, P.G. & Sutton, J.C. 1994b. High temperature, darkness, and drought predispose black spruce seedlings to gray mold. *Canadian Journal of Botany 72*, 135-142.

Zhang, P.G., Sutton, J.C., He, B. & Hopkin, A.A. 1995. Low light intensity predisposes black spruce seedlings to infection by *Botrytis cinerea*. *Canadian Journal of Plant Pathology 17*, 13-18.

Zhang, P.G., Sutton, J.C., Tan, W. & Hopkins, A.A. 1996. *Gliocladium roseum* reduces physiological changes associated with infection of black spruce seedlings by *Botrytis cinerea*. *Canadian Journal of Plant Pathology 18*, 7-13.

Ziogas, B.N., Markoglou, A.N. & Malandrakis, A.A. 2003. Studies on the inherent resistance risk to fenhexamid in *Botrytis cinerea*. *European Journal of Plant Pathology 109*, 311-317.

Zocher, R., Keller, U. & Kleinkauf, H. 1982. Enniatin synthetase: A novel type of multifunctional enzyme catalyzing depsipeptide synthesis in *Fusarium oxysporum*. *Biochemistry 21*, 43-48.

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