Stellingen:

1. Toepassing van *Ulocladium atrum* tegen *Botrytis* spp. is een vorm van ziektepreventie.

   Dit proefschrift.

2. Een goede timing van *Ulocladium atrum* toepassing is essentieel voor succesvolle bestrijding van *Botrytis* spp. en dient gerelateerd te worden aan de fenologie van het gewas.

   Dit proefschrift.

3. De ecologische niche van een competitieve antagonist overlapt in belangrijke mate met die van het te bestrijden pathogeen.

4. Het plukken van door *Botrytis* aangetaste aardbeien is, door het saniterend effect, winstgevender dan het plukken van een zelfde aantal gezonde aardbeien.

5. De ontwikkeling van biologische bestrijdingsstrategieën is maatwerk.

6. Om het gedrag van schimmels te begrijpen moet je 'denken' als een schimmel.

7. Carpoolen verkort de spits maar maakt die, door werktijdsynchronisatie, niet per se minder druk.

8. Het internet is het medium bij uitstek om informatie over RSI te verspreiden.

9. Het World Wide Web heeft meer overeenkomst met een mycelium dan met een web.

10. Het instellen van een vierdaagse werkweek, waarbij de werkdagen over alle dagen van de week worden verdeeld, is effectiever tegen files dan het voortdurend aanpassen van het wegenstelsel.

11. Hallucinerende paddestoelen, wat een gezwam!

(Utrechts Universiteitsblad on-line)

Stellingen behorend bij het proefschrift:
Biological control of Botrytis spp. by Ulocladium atrum
- an ecological analysis -

Wageningen, 1 december 1999 Geert Kessel

Biological control of *Botrytis* spp. by *Ulocladium atrum*

– an ecological analysis –

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Biological control of *Botrytis* spp. by *Ulocladium atrum*

– an ecological analysis –

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Dr C.M. Karssen,
in het openbaar te verdedigen
op woensdag 1 december 1999
des namiddags te vier uur in de Aula

Kessel, G.J.T., 1999

Biological control of *Botrytis* spp. by *Ulocladium atrum*, an ecological analysis.
PhD Thesis Wageningen University, Wageningen, the Netherlands.
- With ref. - With summary in Dutch.

ISBN 90-5808-149-4

Subject headings: *Botrytis* / *Ulocladium atrum* / control / mycelium

The research described in this thesis was conducted at the Research Institute for Plant Protection (IPO), P.O.Box 9060, 6700 GW, Wageningen, the Netherlands and the Laboratory for Theoretical Production Ecology, Wageningen University, P.O.Box 430, 6700 AK, Wageningen, the Netherlands.

This work was financially supported by the Technology Foundation, P.O.Box 3021, 3502 GA, Utrecht, the Netherlands through project WBI44.3284.

Author's abstract

Kessel, G.J.T., 1999. Biological control of *Botrytis* spp. by *Ulocladium atrum*, an ecological analysis. PhD Thesis, Wageningen University, the Netherlands, 155 pp., 12 tables, 18 figures, English and Dutch summary.

Plant pathogenic fungi from the genus *Botrytis* cause economically important diseases in a wide range of crops during the production phase as well as post harvest phase. Control is based on the frequent use of fungicides. Alternative approaches for control are studied because of the development of fungicide resistance in the pathogen and environmental concerns. The fungal saprophytic antagonist *Ulocladium atrum* is an effective biological control agent of *B. cinerea* in cyclamen. *U. atrum* may also be effective against *B. cinerea* in other crops and against other *Botrytis* spp. The biocontrol effect of *U. atrum* is based on a competitive interaction with *B. cinerea* in plant tissue. An immuno-histological technique was developed to visualize the interaction between the mycelia of *Botrytis* spp. and *U. atrum* in plant tissue and quantify colonization levels. Nutrient competition was found to be the dominant antagonistic mechanism between the species and pre-emptive colonization of plant tissue by *U. atrum* was identified as the most promising biocontrol strategy. Necrotic plant tissue is the primary target for *U. atrum* applications, since this is the (only) niche available to both *Botrytis* spp. and *U. atrum*. Pre-emptive colonization of naturally necrotic tissue by *U. atrum* was highly effective against *B. cinerea* in cyclamen, whereas *U. atrum* applications against *B. elliptica* in lily were ineffective. A comparison of both pathosystems suggests three criteria that govern the potential success of *U. atrum* as a biocontrol agent. 1) There must be a niche in which *U. atrum* competes with the pathogen; 2) The competitive ability of *U. atrum* in this niche has to be sufficient to allow for competitive exclusion of the pathogen. 3) The niche from which the pathogen can be excluded has to be essential for disease development. Colonies of *U. atrum* have lower radial growth rates than those of the *Botrytis* spp. studied. In practical biocontrol, this competitive disadvantage of the antagonist can be overcome by applying a high density of its conidia in a uniform distribution on the target tissue.

Voorwoord

Schimmels en andere micro-organismen zijn een essentieel onderdeel van ons dagelijks leven. Het benutten van natuurlijke interacties tussen micro-organismen om plantenziekten te bestrijden is een relatief nieuw terrein van onderzoek. Enkele van de onderzoeksstapjes die nodig zijn om een biologisch alternatief te kunnen bieden voor chemische bestrijdingsmiddelen zijn beschreven in dit proefschrift.
De enorme hoeveelheid werk die verricht is om enig inzicht te krijgen in de interactie tussen plantpathogene *Botrytis* soorten en de antagonist *Ulocladium atrum* komt zeker niet alleen voor mijn rekening. De begeleiding was goed verzorgd, maar liefst vier begeleiders stuurden het onderzoek terwijl twee maal per jaar de STW gebruikerscommissie zijn licht liet schijnen op de behaalde resultaten.
Nyckle Fokkema, jouw ideeën vormden de basis voor het onderzoek aan biologische bestrijding van *Botrytis*. Je hebt je niet dagelijks met het onderzoek bemoeit maar je was beschikbaar als het nodig was. Jouw enthousiasme en inzichten zijn erg belangrijk voor me geweest. Jürgen Köhl, jij verzorgde veelal de dagelijkse sturing van het (praktische) werk en de inbedding en ondersteuning van dit onderzoek binnen de Botrytis groep op het IPO. Menig keer heb jij naar ons geluisterd als er weer eens iets anders liep dan verwacht. Oplossingen voor problemen waren ook vaak van jouw hand. Samen met Wopke van der Werf had je ook de twijfelachtige eer de eerste versie van menig manuscript te mogen bewonderen. Ik ben je zeer erkentelijk voor je onvermoeibare inzet bij het onderzoek wat geleid heeft tot dit proefschrift. Wopke, jij hebt mijn handel en wandel op het voor mij nieuwe gebied van systeem analyse en simulatie in de juiste richting gestuurd. Gedurende de laatste paar maanden van het schrijfwerk zat je in de Verenigde Staten. Dat was echter absoluut geen rem op het correctie werk. Ik had af en toe het idee dat je helemaal nooit sliep terwijl de e-mail verbinding roodgloeiend moest zijn van alle heen en weer gemailde files. Veel dank voor de enorme inzet. Rudy Rabbinge, jij hield strak de hand aan het tijdschema en je hebt ook menig manuscript voorzien van kritische kanttekeningen. Toen ik na anderhalf jaar werk in mineur kwam melden dat Ulocladium waarschijnlijk nooit effectief zou zijn tegen Botrytis elliptica in lelie, was jouw opgewekte commentaar dat ik dan mijn proefschrift kon gaan afronden. Jouw enthousiasme en de energie die er van uitging was erg motiverend.
Heel veel praktisch werk is gedaan door Carin Lombaers - van der plas en Lia de Haas. Jullie inzet en doorzettingsvermogen zijn voor mij een voorbeeld geweest.
Binnen de groep van de bovengrondse ecologen op het IPO was het altijd erg goed toeven. Daarnaast was er altijd hulp bij "noodgevallen". Thijs, Wilma, Helen, Pedro en Pieter, bedankt! De zelfgemaakte slingers zal ik in elk geval nooit vergeten. Daarnaast hebben ook de proefvelddienst, kassendienst, AIV en vele anderen op het IPO bijgedragen aan de totstandkoming van dit proefschrift. Ali wil ik speciaal

bedanken voor de vele vroege koffie en de koeken. Rob Nelissen en Maurice Claassens, jullie hebben als stagiare bijgedragen aan het onderzoek. Jullie kunnen je bijdragen in dit proefschrift terugvinden.
Buiten Wageningen werd ondersteuning gegeven door Ernst van den Ende en Ineke Pennock - Vos van het LBO. Vele leliebollen vonden hun weg naar Wageningen om hier mishandeld te worden met Botrytis. Jullie hulp, enthousiasme, opstekers en adviezen zijn zeer gewaardeerd. De medewerkers van Schoneveld b.v. In Twello wil ik bedanken voor hun niet aflatende enthousiasme voor het Ulocladium werk.
Molley Dewey, your enthusiasm and contributions to our work were very motivating and greatly appreciated. Collaborating with you and Natasha Karpovich was a joy!
I would like to thank all the participants in the PhD discussion group 1 of the C.T. de Wit Graduate School for Production Ecology for their helpful comments on early versions of the manuscripts. Your contributions were incorporated in this thesis.
Tijdens de afronding van het proefschrift heb ik veel hulp gehad van Raph de Rooij. Raph, jouw kennis van DTP gerelateerde zaken was fenomenaal. Ik hoop dat je tevreden bent met het resultaat. Daarnaast waren er ook nog twee kaboutertjes die op miraculeuze wijze onze weekenden thuis citroentjes fris wisten te ontlasten. Het was altijd erg prettig thuiskomen op vrijdagavond.
Mijn ouders wil ik bedanken voor alle stimulansen die ze me gegeven hebben tijdens mijn gehele opleiding. Ik hoop dat jullie op deze manier een klein beetje zicht krijgen op mijn Wageningse werk. Ik draag dit proefschrift aan jullie op.
Tot slot wil ik Rina bedanken. De afgelopen maanden waren niet gemakkelijk en menig weekend en uitstapje is er bij ingeschoten. Zonder jouw hulp en steun had ik het niet gered. Je hebt er wel een bere-conditie aan overgehouden zoals ik recent ondervonden heb. Vanaf nu mag jij weer met de auto naar je werk en ga ik weer fietsen in het weekend, dan kan ik je tenminste weer bijhouden!

Geert

Voor mijn ouders

Contents

Chapter 1

General introduction

General Introduction

Plant pathogenic *Botrytis* spp. are ubiquitous fungi causing economically important diseases in a wide range of host species throughout the world. Control of *Botrytis* diseases is based on the frequent use of fungicides but remains difficult. Biological control of *Botrytis* spp. is considered to be a valuable alternative or supplement to other control measures. In recent years the saprophytic fungal antagonist *U. atrum* was shown to be an effective biological control agent of *Botrytis* spp. The biocontrol effect of *U. atrum* against *Botrytis* spp. is based on both species interacting in necrotic plant tissue. The research presented in this thesis studies the interaction of *U. atrum* and *Botrytis* spp. in necrotic cyclamen- and lily tissue to gain insight in the possibilities of biological control at tissue level, leaf level and crop level.

Plant pathogenic *Botrytis* spp.

Necrotrophic *Botrytis* spp. cause economically important diseases during the production phase as well as post-harvest. Field and greenhouse vegetables, small fruits, ornamentals, flower bulbs and forest tree seedlings are all attacked by *Botrytis* spp. (Coley-Smith et al., 1980). The symptoms include rotting of above ground plant parts and the harvestable- or harvested product. Members of the genus *Botrytis* are commonly classified in two groups. 1) *B. cinerea*, a broad spectrum pathogen which attacks a wide range of host plants and 2) species morphologically distinct from *B. cinerea* which are more specialized in their parasitism (Wood, 1962) e.g. *B. elliptica* on *Lilium* spp. and *B. aclada* on onion. Hyphal cells of *Botrytis* spp. are multinucleate and frequently heterokaryotic. Sexual recombination and heterokaryosis control the heritable variation found in *Botrytis* spp. and generate the genetic potential of *Botrytis* spp. to adapt to changes in the environment (Lorbeer, 1980).
Botrytis cinerea Pers. ex Pers. and *Botryotinia fuckeliana* (de Bary) Whetz. (*Ascomycetes, Sclerotiniaceae*) are the asexual (anamorphic) and sexual (teleomorphic) stages in the life cycle of the same heterothallic filamentous fungus (Faretra et al., 1988). *B. cinerea* is pathogenic to a range of over 200 host plant species (Jarvis, 1977; 1980a) and a saprophyte on necrotic plant material.
Infection of the host can arise from germinating conidia and ascospores, from mycelium growing in dead plant parts and from mycelium established in extraneous organic material (Verhoeff, 1980). The presence of water and exogenous nutrients

is known to enhance infection by *B. cinerea* (Chou and Preece, 1968; Mansfield and Deverall, 1974; van den Heuvel, 1981; Fourie and Holz, 1998). However, conidia can also be infective after dry inoculation and in the absence of exogenous nutrients (Cole et al., 1996). The role of mycelial infections in *B. cinerea* epidemiology is not clearly established but they were shown to play a role in strawberry (Jarvis, 1962d) and in apricot (Yarwood, 1948).
Conidia, ascospores, mycelia and sclerotia can be dispersed by wind, rain splash or growers activities (Jarvis, 1962a, 1962b, 1962c; Ellerbrock and Lorbeer, 1977; Hausbeck and Pennypacker, 1991). Conidia are produced in large quantities in infected crops (Nicot et al., 1996) and can be dispersed over large distances. Despite this dispersal potential, Köhl et al (1995a) demonstrated in a field experiment that the majority of the conidial infections originated from conidia produced within a few meters from the infection site.
Sclerotia are generally believed to be the primary survival structures of *Botrytis* spp. Sclerotia can germinate in three ways: myceliogenic, sporogenic or carpogenic, giving rise to new dispersal propagules (Coley-Smith and Cooke, 1971). Ascospores of *B. fuckeliana* have been reported to serve as primary inoculum in bean (Polach and Abawi, 1975), grapevine (Vanev, 1965; Kublistskaya and Ryabtseva, 1970) and conifers (Hainesworth 1949).
B. cinerea causes leaf rot in cyclamen and is a major pathogen in this crop (Nightingale, 1982; Jacob, 1987). *B. cinerea* infection of cyclamen results in expanding lesions on petioles and leaf blades. Contact infections from petiole to petiole in the dense canopy of the cyclamen plant cause the typical symptom of diseased leaf clusters which reduce the ornamental value of the plant.

B. elliptica Berk. Cooke causes destructive leaf infections in lily plants ('leaf fire') resulting in serious yield losses (Doss et al., 1986). The host plant range of *B. elliptica* is practically limited to lily (*Lilium* spp.) although alternative hosts have been reported (MacLean, 1948). Conidia or ascospores causing primary infections of *B. elliptica* were reported to originate from sclerotia present in the soil and on plant debris (van den Ende and Pennock-Vos, 1997). *B. elliptica* is spreading within and between lily plants through contact infections and splash or wind dispersal of conidia (Beyma thoe Kingma and van Hell, 1931). *B. elliptica* infection of lily leaves results in brown expanding lesions. Associated with the lesion, yellow streaks of senescing tissue can be formed, distally and proximally from the lesion (Doss et al., 1988). From the leaves *B. elliptica* migrates to the stem where it can block vascular

transport and kill plant parts located above the location of stem infection (Beyma thoe Kingma and van Hell, 1931).

Chemical disease control

At present, the control of diseases caused by *Botrytis* spp. is based on the frequent use of fungicides. Despite the availability of several effective fungicides and improved application techniques, control of *Botrytis* diseases is often difficult and incomplete (Gullino, 1992). *Botrytis* spp. have demonstrated a great potential to develop fungicide resistance (Gullino, 1992; Migheli et al., 1990). A constant selection pressure exerted by the frequent use of fungicides led to the development of resistance to benzimidazoles in the early 1970's (Bollen, 1982; Delp, 1988) and to dicarboximides in the 1980's (Lorenz, 1988). Resistance to both benzimidazoles and dicarboximides is now widespread (Gullino, 1992). Chemical control strategies for *Botrytis* spp. using fungicides with different modes of action in alternation or combination in order to avoid resistance development are now common practice, but control of *Botrytis* diseases remains difficult (Leroux, 1994). On top of this, increasing concerns about the effects of pesticide residues on environment and human health (Jansma et al., 1993) lead to an increasing number of restrictions on the use of pesticides. Integration of chemical, cultural and biological control measures guided by decision support systems is now becoming a rational and realistic aim to control diseases caused by *Botrytis* spp. (Gullino, 1992; Kerssies, 1994; Nicot and Baille, 1996).

Biological control of necrotrophic fungal pathogens.

For biological control of fungal foliar pathogens using introduced antagonists, usually a distinction is made between necrotrophic and biotrophic pathogens. This distinction is based on the potential of nutrient competition in the antagonistic interaction (Köhl and Fokkema, 1998). Nutrient competition will only be active against necrotrophic pathogens and not against biotrophic pathogens because biotrophic pathogens do not depend on exogenous nutrients or necrotic plant tissue for infection and escape from interaction with antagonists in the phyllosphere after infection. Infections by necrotrophic pathogens on the other hand are dependent on or stimulated by exogenous nutrients (e.g. Chou and Preece, 1968; Fokkema et al.,

1983). Necrotrophic pathogens kill healthy plant tissue by means of toxins or enzymes before they colonize the tissue and utilize the available resources.
Three strategies for biological control of necrotrophic pathogens can be distinguished (Fokkema, 1993; Köhl and Fokkema, 1998):

- microbial suppression of infection,
- microbial suppression of mycelial colonization, survival and sporulation of the pathogen in necrotic plant tissue,
- microbial degradation of sclerotia as the primary inoculum for future crops.

The choice for a biological disease control strategy depends on the sensitivity of the pathogen and the efficiency of the biocontrol agent during the different stages of the pathogens life cycle. The choice for an antagonist depends on the antagonist's suppressive capabilities, its adaptation to the niche in which the interactions take place and on the average interaction time.
Short interaction times between pathogen and antagonist require fast antagonistic mechanisms, such as the production of antibiotics or other compounds toxic to the competitor. Longer interaction times allow the use of antagonists effective through slower antagonistic mechanisms such as nutrient competition. The niche in which the interaction takes place is characterized by nutritional, abiotic and biotic factors. Biocontrol agents introduced into a specific niche need to be ecologically adapted to that niche.

Microbial suppression of infection

Microbial suppression of infection takes place on the leaf surface, where interaction times between pathogen and antagonist are short. This habitat is characterized by rapid fluctuations of temperature, water availability (Burrage, 1971) and nutrient availability. All these factors are determining factors in the population dynamics of the micro-organisms living on the leaf surface. Naturally occurring bacteria, yeasts and fungi populating the phyllosphere depend on short periods suitable for growth and have to be able to survive during periods unsuitable for growth with e.g. low water availability or extreme temperatures. Nutrients available in the phyllosphere include leaf leachates (Tukey, 1971), pollen (Chou and Preece, 1968) and aphid honeydew (Dik et al., 1991). These nutrients also stimulate infection of the leaf by necrotrophic pathogens. Consumption of phyllosphere nutrients by the natural

microbial population neutralizes this stimulation of infection, thus providing a natural mechanism of disease control of necrotrophic pathogens (Dik et al., 1991). Fungicides applied to the leaves can eliminate the natural phyllosphere flora after which phyllosphere nutrients remain on the leaves, thus stimulating infections by necrotrophic pathogens (Fokkema et al., 1975). Other examples of microbial suppression of infection involve the use of introduced micro-organisms producing antibiotics (Knudsen and Spurr, 1987), micro-organisms producing surfactants reducing the leaf wetness duration (Seddon and Edwards, 1993) and the use of *Trichoderma harzianum* isolate T39, which is capable of reducing the level of hydrolytic enzymes produced by *B. cinerea* on the leaf surface (Kapat et al., 1998; Zimand et al., 1996). Trichodex, a commercially available biocontrol product against *B. cinerea* was developed based on *T. harzianum* T39.

Microbial suppression of mycelial colonization and sporulation

Microbial suppression of mycelial colonization during the saprophytic phase of necrotrophic pathogens in necrotic tissue has the advantage of significantly longer interaction times then microbial suppression of infection (Fokkema, 1993). After senescence, plant tissue becomes available for saprophytic colonization by saprotrophic and necrotrophic (pathogenic) micro-organisms. Similar to healthy plant tissue, necrotic tissue is also exposed to extreme and strongly fluctuating climatic conditions (Köhl et al., 1995b). The principal nutrient sources in naturally senesced tissue are formed by remaining cell wall components such as cellulose, hemicellulose and lignin. The amino acids and soluble sugars content is relatively low due to relocation of these substances to healthy plant parts or reproductive organs during senescence (Baddeley, 1971). In plant tissue necrotic due to other causes than senescence, the nutrient sources may include the amino acids and soluble sugars. Primary colonizers of necrotic tissue, competing for the available resources include *Cladosporium* spp., *Alternaria* spp., *Aureobasidium pullulans*, *Epicoccum nigrum* and *B. cinerea* (Hudson, 1971). Competitive exclusion of one primary colonizer by another is a common phenomenon within this group of fungi. This can be illustrated by the incapability of the pathogenic *Fusarium roseum* f. sp. *cerealis* 'Culmorum' to colonize wheat straw which is already colonized by *Penicillium* spp., *Alternaria* spp., *Stemphylium* spp. or *Aspergillus* spp. (Cook, 1970). Competitive interactions between pathogenic and non-pathogenic primary colonizers of necrotic plant tissue again provide a natural level of disease control.

The level of the natural disease control is a function of the available inocula and the environment including the nutritional status of the substrate and micro climate (Andrews, 1992). Examples of biological control via microbial suppression of mycelial colonization and sporulation include application of the fungi *Athelia bombacina* and *Chaetomium globosum* to apple leaves on the orchard floor to decrease ascospore production by *Venturia inaequalis* (Heye and Andrews, 1983) and application of the fungus *Limonomyces roseipellis* to wheat straw to reduce sexual reproduction of the wheat pathogen *Pyrenophora tritici-repentis* (Pfender et al., 1993).

Microbial degradation of sclerotia

Sclerotia are survival structures producing the primary inoculum in future crops. Microbial degradation of sclerotia aims at a reduced survival or viability of pathogenic sclerotia in the soil or on/in crop debris. This approach has the advantage of long interaction times between pathogen and antagonist.
Coniothyrium minitans is a ubiquitous soilborne mycoparasite of plant pathogenic *Sclerotinia* spp. (Winsch et al., 1993; Zhou and Boland, 1998; Whipps and Gerlagh, 1992). Application of *C. minitans* to an infected crop results in a reduction of the primary inoculum for future growing seasons (Gerlagh et al., 1999). *C. minitans* may also be applied to the soil to destroy sclerotia of *Sclerotinia* spp. pathogenic to e.g. lettuce or oil seed rape. This application strategy of *C. minitans* reduces the disease pressure in the current growing season (McQuilken et al., 1995; Budge et al., 1995). The commercially available biocontrol product Contans, which is effective against *S. sclerotiorum* and *S. minor*, is based on *C. minitans*.

Epidemiology

Several factors control the rate at which a disease epidemic progresses: infection efficiency, length of the latent period, lesion growth rate and sporulation capacity. (Zadoks and Schein, 1979). The reduction of one of these factors may by itself not be enough to significantly hamper the development of an epidemic. Compensatory effects have been reported. *B. cinerea* in begonia was able to compensate for low initial inoculum by accelerated rates of disease progression (Plaut and Berger, 1981).

Microbial degradation of the primary inoculum is specifically effective for *Sclerotinia sclerotiorum*, because this pathogen does not use conidia for secondary dispersal. Leonard and Mundt (1984) and Sache and Vallavieille-Pope (1995) both used mathematical models to analyse the relation between each of the factors involved in disease progress and the progress of the epidemic. They both concluded that for species with short latent periods and large sporulation capacities, such as *B. cinerea*, decreasing the sporulation capacity and thus the reproduction per generation would be of great benefit to the crop.

Biological control of *Botrytis* spp. using *Ulocladium atrum*

Ulocladium atrum Preuss (*Deuteromycetes, Dematiaceae*) is a saprophytic fungus, mostly encountered in conjunction with degradation of organic materials, in soils and above ground. It is also one of the micro-organisms responsible for the degradation of wood and stone of monumental buildings (Almendros et al., 1985; Gutierez et al., 1995). In three cases *U. atrum* has been identified as a minor plant pathogen responsible for leaf spot in cucumber (Butler et al., 1979), leaf spot disease of potato in Peru (Turkensteen, 1979) and achene blemish of sunflower in Israel (Shtienberg, 1994). In recent years *U. atrum* isolate 385 was identified as an effective biological control agent of *Botrytis* spp. (Köhl et al., 1995b, 1995c, 1998). *U. atrum* 385 was isolated from a necrotic tip of a field grown onion. It suppresses sporulation of *Botrytis* spp. under greenhouse and field conditions (Köhl et al., 1995b, 1995c). Conidia of *U. atrum* survive long dry periods in the phyllosphere (Köhl et al., 1995c) and *U. atrum* is growing in a temperature range matching that of *Botrytis* spp. (Köhl et al., 1999). In commercial cyclamen crops *U. atrum* applications were as effective against *B. cinerea* as the growers' standard fungicide program (Köhl et al., 1998). The control effect of *U. atrum* against *Botrytis* spp. is based on both species competitively interacting in plant tissue (Köhl et al., 1997).

Mycelial interactions

Biological control through suppression of pathogen development in necrotic tissue is an attempt to manage an interacting microbial community in favour of the biocontrol agents. Biocontrol agents are applied to pre-emptively colonize a substrate or to recolonize a substrate displacing the pathogen (Andrews, 1992). Park

(1960) classified harmless or beneficial fungal interactions as symbiotic and interactions detrimental to at least one of the species as antagonistic. Three antagonistic mechanisms were distinguished: competition, antibiosis and parasitism (Park, 1960, 1968; Clark, 1965; Baker and Cooke, 1974). Another classification for fungal interactions was given by Cooke and Rayner (1984) and focuses on resource capture. They classified mycelial interactions as mutualistic (beneficial to both), neutralistic (beneficial nor detrimental to both) or competitive (detrimental to either or both). Competitive interactions were divided into primary resource capture and combative interactions. Primary resource capture is the process of gaining initial access to, and influence over an available resource. Combative interactions were classified as defensive or offensive. In a defensive interaction access to resources gained by primary capture is denied to other species. During offensive interactions or secondary resource capture, access is gained to an already occupied resource and the established species can be replaced.
In relation to biological control, Cooke and Rayner (1984) distinguish preventative and curative biological control. The principles underlying preventative biological control will usually involve primary resource capture whereas combative interactions and especially secondary resource capture or direct parasitism are characteristic for curative biological control.
Thus, the later the antagonist is applied relative to the pathogen the more aggressive the antagonist needs to be to capture the available resources and achieve the desired biocontrol effect. Biological control through microbial interactions within lesions (Biles and Hill, 1988) thus requires antagonists with a different mode of action then biological control through pre-emptive colonization of a substrate (e.g. Pfender et al., 1993).

Systems analysis, simulation and biological control

Biological control is applied ecology. The interaction between a pathogenic and an antagonistic population as influenced by biotic (host plant and natural microflora) and abiotic conditions (climate and nutritional status of the niche) together determine the result of the biocontrol effort. As a whole, a biocontrol system offers an enormous complexity. Systems analysis and simulation have been used as tools to analyse biocontrol interactions and to bring order in the complexity. Knudsen and Hudler (1987) developed a simulation model to analyse interactions between antagonistic *Pseudomonas fluorescens* and *Gremmeniella abietina*, a leaf pathogen

of *Pinus resinosa*. They demonstrated that populations of the bacterial antagonist were extremely sensitive to dry periods whereas germinating conidia of the pathogen were insensitive to small variations in the bacterial concentrations. Based on the model results they concluded that the antagonist was unsuitable for application to the phyllosphere and formulated requirements for an effective biocontrol agent. Dik (1991) developed a computer simulation model for the consumption of aphid honeydew by phyllosphere yeasts. The model was used to assess the effect of yeasts on the amount of honeydew as a stimulant for infection by necrotrophic pathogens under different scenario's of aphid and yeast populations as influenced by fungicides. This resulted in quantitative predictions of the role of the natural phyllosphere population as antagonists against necrotrophic pathogens. For the current research, systems analysis was used as a tool to identify key aspects of the *Botrytis* spp., *U. atrum* interaction at tissue level and plant and crop level. Simulation was used to integrate the knowledge generated and evaluate its consequences for system dynamics and biological control.

Research target

The aim of the research described in this thesis was to identify the key ecological principles underlying biological control of *Botrytis* spp. using *Ulocladium atrum* and to elucidate the mechanisms of competition between *Botrytis* spp. and *U. atrum* in plant tissues. In turn, this will contribute to the development of reliable biocontrol strategies against *Botrytis* spp. *Ulocladium atrum* was chosen as a biocontrol agent because of its proven capability to suppress *Botrytis* spp. in necrotic tissue under interrupted leaf wetness periods (Köhl et al., 1995b) and under field conditions (Köhl et al., 1995c). *Botrytis elliptica* and *Botrytis cinerea* were chosen as a specialized and broad spectrum pathogen respectively. *Botrytis* spp. and *U. atrum* interact in necrotic tissue but knowledge of the competitive interaction between *B. cinerea* and *U. atrum* is limited. A thorough knowledge of competitive substrate colonization by *B. cinerea* and *U. atrum* is essential to understand the possibilities and limitations of the antagonist and to identify options to manipulate the interaction in favour of the antagonist.

Outline of this thesis

This thesis addresses several aspects of biological control of *Botrytis* spp. using the antagonist *U. atrum*. Chapter 2 describes the development of a technique to visualize and to quantify the volume of interacting mycelia of *Botrytis* spp. and *U. atrum* in necrotic plant tissue. This technique allows investigation of spatio-temporal aspects of competitive substrate colonization by fungi. It is applied in Chapter 3 to study temporal, spatial and mechanistic aspects of competitive colonization of necrotic cyclamen leaves by *B. cinerea* and *U. atrum*. This results in the identification of the ecological principles underlying suppression of colonization and sporulation.
Chapter 4 describes a study in which successful biocontrol of *B. cinerea* in cyclamen by *U. atrum* is compared to unsuccessful biocontrol of *B. elliptica* in lily by the same antagonist. In a comparative approach ecological key factors that determine the biocontrol potential of *U. atrum* at plant and crop level are derived. In Chapter 5 a spatially explicit dynamic simulation model of the interaction between *B. cinerea* and *U. atrum* in necrotic cyclamen leaf tissue is described. This model integrates the information presented in the previous chapters and is used to study the general behaviour of the system and to identify knowledge gaps. Chapter 6 discusses scientific and applied aspects of the research presented. Methodological aspects of the current research are discussed and possibilities for future work are indicated.

Chapter 2

Quantification of mycelium of *Botrytis* spp. and the antagonist *Ulocladium atrum* in necrotic leaf tissue of cyclamen and lily by fluorescence microscopy and image analysis

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Phytopathology 89: 868 - 876.

Abstract

A technique was developed to localize and quantify the internal mycelial colonization of necrotic leaf tissue of cyclamen (*Cyclamen persicum*) or lily (*Lilium*) by pathogenic *Botrytis* spp. and the antagonist *Ulocladium atrum*. This technique allows investigation of competitive substrate colonization by both fungi which is a key process for biological control of *Botrytis* spp. by *U. atrum*. A combination of differential fluorescent labelling and image analysis was applied on cryostat sections of necrotic leaf tissue. *Botrytis* mycelium was labelled specifically by indirect immunofluorescence using a monoclonal antibody specific for *Botrytis* spp. and an anti-mouse fluorescein conjugate. Wheat germ agglutinin conjugated to the fluorochrome TRITC was used to label mycelium of both fungi. Image analysis was used to measure the relative surface area of the cryostat section covered by fluorescing hyphae of *Botrytis* spp. and by fluorescing hyphae of both fungi. A mathematical conversion was derived and used to calculate the relative mycelial volume of each fungal species in the necrotic tissue based on the measured relative surface areas. Temporal aspects of substrate colonization were studied in a short time series. An analysis of components of variance provided some insight into spatial colonization patterns for the fungal species involved and allowed the design of efficient sampling strategies for future experiments.

Introduction

Botrytis spp. cause economically important diseases in numerous greenhouse and field crops. In lilies (*Lilium*), *B. elliptica* Berk. Cooke can cause destructive leaf infections ('leaf fire') resulting in serious yield losses (Doss et al., 1986). In cyclamen (*Cyclamen persicum*), *B. cinerea* Pers.: Fr. causes leaf rot and is considered to be one of the major pathogens in this crop. At present, the control of *Botrytis* diseases depends on the frequent use of fungicides. However, *Botrytis* spp. have shown a great potential to develop fungicide resistance (de Waard et al., 1993; Gullino, 1992). The threat of fungicide resistance and an increasing number of governmental restrictions on the use of pesticides has increased the need for alternative control measures. Biological control offers an environmentally friendly supplement or alternative to chemical control.

Necrotrophic pathogens such as *Botrytis* spp. kill plant tissue before invading it and sporulate exclusively on necrotic tissue. The saprophytic fungal antagonist *Ulocladium atrum* Preuss isolate 385 suppressed sporulation of *Botrytis* spp. in several necrotic plant tissues (Köhl et al., 1998; Köhl et al.,1995b; Köhl et al.,1995c). As demonstrated for *Botrytis* spp. in onion, reduction of the sporulation of the pathogen causes a delay in the disease build up (Köhl et al., 1995a). The mechanism by which *U. atrum* suppresses sporulation of *Botrytis* spp. is unknown but Köhl et al. (1997) suggested that toxins and direct parasitism were not involved. Antagonism is probably due to nutrient competition.

Competitive substrate colonization by the pathogen and the saprophytic antagonist resulting in reduction of sporulation takes place within the necrotic plant tissue from the earliest phases of colonization onward. Detailed knowledge of the spatial and quantitative characteristics of the competitive colonization of necrotic plant tissue by these fungi is missing and techniques to study this subject are, to the best of our knowledge, not available. Quantification of the spore producing surface area (Elad et al., 1994a; Köhl et al.,1995b), conidial production (Biles and Hill, 1988) or fruiting body formation (Adee et al.,1990; Newton et al., 1997) does not give information about the early stages of colonization or the extent of internal colonization of the tissue. Chemical techniques quantifying fungal constituents such as chitin or ergosterol (Hicks and Newell, 1983; Lumsden et al., 1990; Ride and Drysdale, 1972; Seitz et al., 1979) are not very sensitive and cannot selectively quantify specific fungal populations (Lumsden et al., 1990). Immunological (Bermingham et al. 1995; Dewey et al., 1992; Harrison et al., 1990; Karpovich-Tate et al., 1998; Plasencia et al., 1996; Ricker et al., 1991) or molecular methods (Hu et al., 1993) can quantify the total (i.e. internal plus external) fungal biomass. Furthermore chemical, immunological and molecular techniques rely on homogenization of the colonized substrate which makes them inappropriate to study spatial aspects of fungal colonization.

A thorough knowledge of competitive substrate colonization will result in identification and understanding of the possibilities and limitations of the antagonist. This knowledge is essential to predict when antagonist applications will result in successful disease control and when other control measures should be used. The objective of this study was to develop a technique to quantify internal colonization of necrotic cyclamen tissue by *B. cinerea* and *U. atrum* and of necrotic lily tissue by *B. elliptica* and *U. atrum*. This was achieved using an immuno-histological approach. Our method has the advantage of being able to provide both quantitative and qualitative data on two fungal species competitively colonizing necrotic plant tissue.

Development of the procedure involved optimization of methods for sectioning necrotic cyclamen and lily tissue, optimization of dual fluorescent fungal labelling techniques and development and optimization of an image analysis program. Variance components for the different sampling levels were estimated and used to design optimal sampling strategies for the experiments. As a final step, a mathematical conversion was derived which relates the measured relative surface area of the section covered by fungal hyphae to the volume of hyphae within the section as an estimator for fungal biomass.

Materials and methods

Cultures

Botrytis cinerea, isolate 700, was originally isolated from a diseased gerbera (*Gerbera jamesonii*). Isolate BE9401 of *Botrytis elliptica*, isolated from a diseased lily, cv Mont Blanc, was provided by E. van den Ende (Bulb Research Centre (LBO), Lisse, the Netherlands). *Ulocladium atrum* isolate 385 was isolated from a necrotic leaf tip of a field grown onion (Köhl et al., 1995c). *B. cinerea* and *B. elliptica* were stored as conidial suspensions in 15% glycerol at -80°C. *U. atrum* was maintained on oatmeal agar slants (20 g of milled oats, 15 g of agar (Oxoid L13, Unipath Ltd, Basingstoke, UK) and 1 L of tap water) at 4°C. Before use, *B. cinerea* was cultured on oatmeal agar for two weeks at 20°C in the dark. *B. elliptica* was cultured on malt extract agar (Oxoid CM59) for 2 weeks at 18°C under the continuous combined light of an 18W black light and an 18W cool-white fluorescent tube suspended 20 cm above the cultures. *U. atrum* was cultured on oatmeal agar for 4 weeks at 18°C in the dark. Conidial suspensions of all fungi were prepared by flooding the cultures with sterile tap water containing 0.01% Tween 80. After gently rubbing with a rubber spatula to detach the conidia, suspensions were filtered through nylon gauze with a mesh of 200 µm. Conidial concentrations were determined using a haemocytometer and adjusted with sterile tap water containing 0.01% Tween 80 to $1x10^4$ conidia/ml for *B. elliptica* and $1x10^6$ conidia/ml for *B. cinerea* and *U. atrum*.

Plant material and leaf inoculation

Cyclamen plants (*Cyclamen persicum* L.) cv. Superserie were grown in a greenhouse set at 18°C during the autumn without additional light. Flower buds were removed to prevent pollen deposition on the leaves which is known to enhance infection by *B. cinerea* (Chou and Preece, 1968). Mature healthy leaves were removed from the plants and dried slowly at room temperature to simulate natural senescence as closely as possible (Köhl et al., 1995c). After drying the petiole was removed, the leaf was cut in half along the midvein and sterilized by gamma irradiation (4 Mrad, cobalt-60 gamma source, Gammaster BV, Ede, the Netherlands). Before inoculation, leaves were rehydrated in sterile tap water. Excess water was removed by blotting the leaves on dry sterile filter paper. Subsequently, each leaf was placed on top of two pieces of sterile filter paper (50 mm in diameter) containing 0.6 ml of sterile water in sterile plastic petri dishes (55 mm in diameter, one halved leaf per dish). Leaves in the petri dishes were sprayed with a conidial suspension of *B. cinerea* ($1x10^6$ conidia/ml), a conidial suspension of *U. atrum* ($1x10^6$ conidia/ml), conidial suspensions of both fungi at the same concentrations or sterile tap water containing 0.01% Tween 80 using an atomizer (Desaga, Heidelberg, Germany). When appropriate, *U. atrum* was applied directly after the application of *B. cinerea*. This resulted in conidial densities of 26 ± 8 conidia/mm^2 for *U. atrum* and 23 ± 11 conidia/mm^2 for *B. cinerea*. Sprayed leaves were incubated at 18°C in the dark.
Asiatic hybrid lilies (*Lilium*) cv. Mont Blanc were grown in pots in the greenhouse at 18°C with 16 h of light per day. Flower buds were removed, as is usual in bulb production and to prevent pollen deposition on the leaves. Healthy leaves, approximately 8 cm long, were removed from 3 months old plants, dried at 60°C for 48 h and sterilized by gamma irradiation (4 Mrad). Before inoculation, leaves were washed three times in sterile tap water to remove soluble nutrients. Excess water was removed by blotting the leaves on dry sterile filter paper. Subsequently, leaves were placed on top of two pieces of sterile filter paper (80 mm in diameter) containing 1.5 ml of sterile water in sterile plastic petri dishes (90 mm in diameter, four leaves per dish). Leaves in the petri dishes were sprayed with both a conidial suspension of *B. elliptica* ($1x10^4$ conidia/ml) and a conidial suspension of *U. atrum* ($1x10^6$ conidia/ml) or sterile tap water containing 0.01% Tween 80 using an atomizer. *U. atrum* was applied 8 h after the application of *B. elliptica*. Four petri dishes were sprayed per treatment. Sprayed leaves were incubated at 18°C under the continuous combined light of an 18W black light and an 18W cool-white fluorescent tube suspended 20 cm above the petri dishes.

Preparation, sectioning and staining of colonized leaves

Lily or cyclamen leaf tissue samples approximately 3 x 5 mm in size were cut using a scalpel and placed in an eppendorf vial containing 1 ml 3% (w/v) paraformaldehyde in PBS (phosphate buffered saline solution) for overnight fixation. Samples from the same leaf were placed in the same eppendorf vial. After fixation leaf samples were transferred to a graded series of sucrose solutions with increasing concentrations of 5, 10, 20, 30, 40 and 50 % (w/v) in PBS. Samples were kept in each concentration for ½ to 1 hour. Samples were blotted on dry filter paper to remove excess sucrose solution before embedding in Tissue-Tek O.C.T. compound (Miles, Elkhart, IN). Cross sections, 8 µm in thickness for cyclamen leaves and 16 µm in thickness for lily leaves, were cut using a cryostat (Microm, HM 500 O, Microm Laborgeräte GmbH, Walldorf, Germany) at -30°C. Tissue sections were transferred to poly-l-lysine (Sigma Chemical Co, St. Louis, MO, p-1524; 0.1 % in milliQ (w/v)) coated slides and dried on a slide drying bench at 50°C for 15 minutes.

Hybridoma supernatant from cell line BC-KH4 (Bossi and Dewey, 1992) was used to selectively detect *B. elliptica* hyphae in lily tissue sections and *B. cinerea* in cyclamen tissue sections. The monoclonal antibody (MAb) BC-KH4 was originally raised against a *B. cinerea* isolate from grapes and is considered to be genus specific (Cole et al., 1996). Wheat germ agglutinin conjugated to tetramethylrhodamine isothiocyanate (WGA-TRITC) was used to label both fungi in lily and cyclamen tissue sections. This lectin exhibits a specificity for hyphal fragments (Morell et al., 1985) and selectively binds to chitin.

Dual fluorescent labelling of the hyphae in the tissue sections on the glass slides was carried out according to the following schedules.

Slides with cyclamen tissue sections were rinsed in PBS containing 0.1% (v/v) Tween 20 (PBST) for 10 minutes to remove the embedding material. Subsequently, 2% (w/v) bovine serum albumin (Merck, Darmstadt, Germany, Art. 12018) + 1% (v/v) normal goat serum in PBS was applied for 30 minutes to block binding to nonspecific sites. The slides were then rinsed two times in PBST for 15 minutes. The MAb BC-KH4 (diluted 1:5 in PBST + 0.1% (w/v) acetylated bovine serum albumin: PBS-BSA-C (Aurion, Wageningen, the Netherlands)) was then applied and incubated overnight at 4°C. After incubation, tissue sections were rinsed three times in PBST for 30 minutes. FITC-conjugated µ-specific goat-anti-mouse IgG (1020-02, Southern Biotechnology Associates. Al.) (diluted 1:80 in PBST-BSA-C) and the nonspecific fluorescent stain WGA-TRITC (Sigma L-5266; 0.033 mg protein/ml PBS-BSA-C) were mixed in equal volumes and applied to the sections which were then incubated for

one hour at 20°C. Sections were again rinsed three times in PBST, mounted in citifluor anti quench (glycerol in PBS: Citifluor Limited, London, UK), and covered with a cover slip. Treated samples were stored at 4°C in the dark for later examination.
Slides with lily tissue sections were rinsed in PBST for 10 minutes to remove the embedding material. Subsequently, a 0.3% casein (Sigma C-5890) solution in PBS was applied for 30 minutes to block binding to nonspecific sites. The slides were then rinsed twice in PBST for 5 minutes and twice in milliQ. The MAb BC-KH4 (diluted 1:5 in PBST + 0.1% (w/v) BSA: PBST-BSA) was then applied and incubated overnight at 4°C. Tissue sections were rinsed twice in PBST and twice in milliQ. FITC-conjugated goat-antimouse IgG (Sigma F-9006) (diluted 1:10 in PBST-BSA) and the nonspecific fluorescent stain WGA-TRITC (Sigma L-5266; 0.033 mg protein/ml PBST-BSA) were mixed in equal volumes, applied to the sections and incubated for one hour at 20°C. Sections were again rinsed twice in PBST and twice in milliQ and treated further as described for cyclamen tissue sections.
Differences in the staining procedure for cyclamen and lily tissue sections were due to higher levels of background fluorescence encountered in cyclamen tissue. Acetylated BSA (BSA-C) was used in the cyclamen protocol because it is highly efficient in suppressing charge determined background fluorescence (van de Plas and Leunissen, 1993).

Microscopy

Stained tissue sections were examined with a Leica DM RB fluorescence microscope (Leica, St. Gallen, Switzerland) equipped with three filter sets housed in a rotating carrier. An "HQ" filter block (excitation 535/emission 610) was used for TRITC detection (Fig. 2.1A), an "L4" filter block (BP450-490/RKP510/BP515-560) was used for FITC detection (Fig. 2.1B) and an "A" filter block (BP340-380/RKP400/LP430) was used to obtain an image of the section based on autofluorescence only (Fig. 2.1C). Fungal hyphae were not visible using the "A" filter block. This filter did however provide a clear 'background' image of the section.
Digitized images and photographs of the sections of colonized tissue were obtained at 200x magnification. Ektachrome ISO 100/21° film (Kodak, Rochester, NY) was used for the photographs.

Image analysis

The image analysis package MicroGop 2000S (Context Vision, Kista, Sweden) was used to automatically detect and estimate the surface area covered by each of the two dually labelled fungi. Using the tools of this package a dedicated program was developed on a SPARC 5 workstation operating under Solaris 2.4 using X-WINDOWS as a graphical interface (SUN Microsystems, Inc., Mtn. View, CA).
The images were obtained from a low-lux Black and White cooled CCD camera PLT450 (resolution: 604x576 pixels; HCS Vision Technology, Son, the Netherlands) attached to the binocular tube of the Leica DM RB fluorescence microscope through a C-mount adapter and a 0.3x phototube. This camera produces low noise images under poor light conditions (fluorescence) by means of integration techniques. An S-Bus frame grabber DASM-VIP (Analogic Corp., Peabody, Massachusetts) connected with the SPARC-Bus (SCSI) was used for acquisition of the images.
The image analysis program consisted of a loop with a dialogue part for interaction with the user, grey scale image processing, thresholding, binary image processing and display functions. This loop had to be completed for each section that was analysed.
The loop started with the dialogue section in which the grey value TRITC (Fig. 2.1D), FITC (not shown) and background image (not shown) were obtained using the HQ, L4 and A filter block respectively. The user then draws a fine line along the edges of the section in the grey value background image so that an accurate estimate for the surface area of the section is obtained. The detected surface area of the section was contained in a binary image (not shown).
In the grey scale image processing step, the grey-value background image was subtracted from the grey value TRITC (Fig 2.1D) and grey value FITC images, respectively. This resulted in corrected grey value TRITC (Fig. 2.1E) and FITC (not shown) images that almost exclusively contained the brightly fluorescing structures, representing the fungal hyphae, without the original background. The corrected grey value TRITC and FITC images were thresholded resulting in estimates for the fungal surface area contained in two binary images: a binary TRITC image (red overlay in Fig. 2.1F) and a binary FITC image (green overlay in Fig. 2.1F which was placed on top of the red TRITC overlay). The threshold value was set manually in the first section of a batch of sections to assure that the detected fungal area represented the fungal area, as it is seen through the microscope, as closely as possible. The selected threshold values for FITC and TRITC detection were then stored and used for the

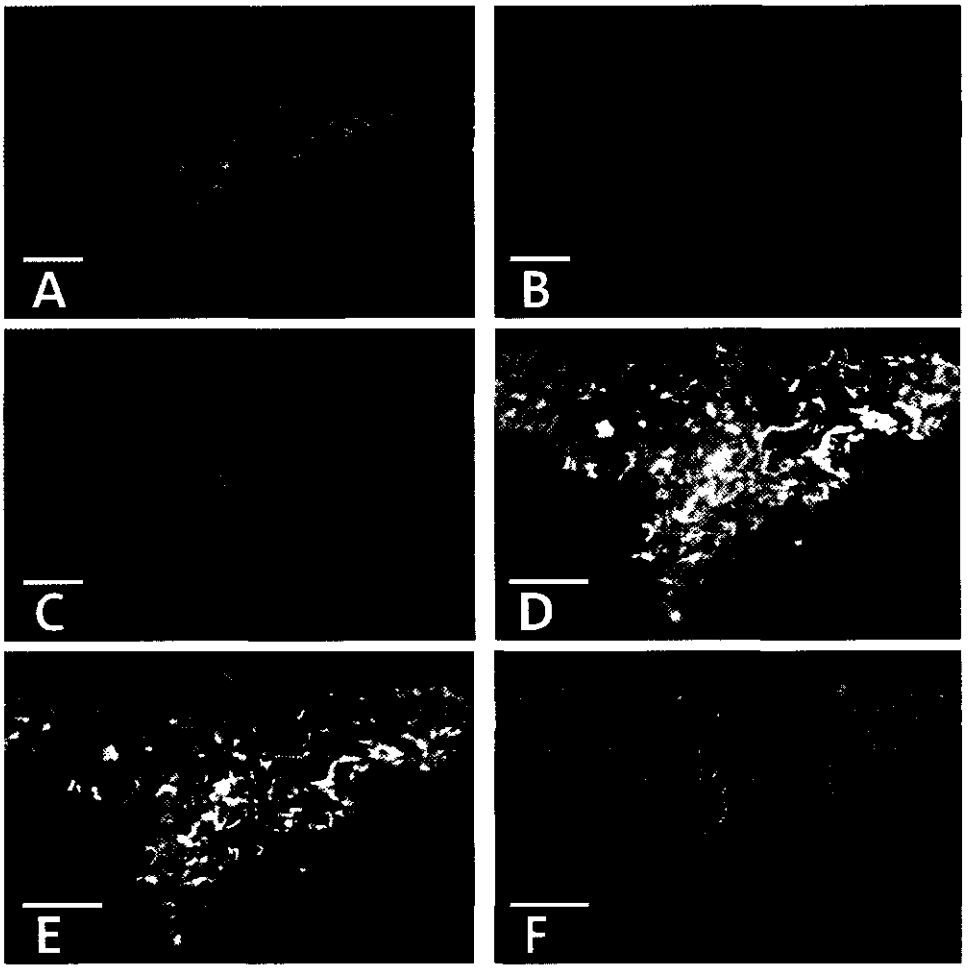

Figure 2.1. Photographs (**A**, **B** and **C**) and digitized images (**D**, **E** and **F**) of a cross section of a necrotic lily leaf colonized by *B. elliptica* and the antagonist *U. atrum* 51 hours after inoculation. **A**: all fungi present in the section were labelled using wheat germ agglutinin conjugated to the fluorochrome TRITC. **B**: *B. elliptica* is selectively labelled using the MAb BC-KH4 conjugated to the fluorochrome FITC. **C**: Background image of the section as seen through a Leica "A" filterblock which does not show the fluorochromes TRITC and FITC. **D**: grey value image of "**A**". **E**: grey value image calculated by subtracting the grey value image of **C** from **D** resulting in a separation of fungal hyphae from the original background present in **D**. **F**: Composite image with the total fungal area detected (red) on top of image **E** and the detected *B. elliptica* area (green) on top of both **E** and the total fungal area detected. As a result of this layering *B. elliptica* is represented by the green areas and *U. atrum* is represented by the red areas. Bars represent 100 μm.

whole batch of prepared sections. This procedure allowed a small correction for variation in labelling intensity between batches of stained sections.
In the binary image processing step, the surface area of the whole tissue section, the surface area of the tissue section covered by *Botrytis* spp. and the surface area of the section covered by both fungal species were measured from the binary background, binary FITC and binary TRITC images respectively (μm^2). The areal density (fraction of the section covered by hyphae (Weibel, 1979)), was calculated from the measured values and converted into the relative volume, or volume density (Weibel, 1979), of the mycelia of both species in the section using the procedure described below.
In the display procedure *Botrytis* spp. and *U. atrum* hyphae were shown in two separate colour overlays (green and red respectively) on top of the grey value TRITC image thus showing how the *Botrytis* spp. and *U. atrum* hyphae were situated within the leaf (Fig. 2.1F). Values obtained for the relative surface area of *Botrytis*, *U. atrum*, both species and the area of the section were displayed and stored on file.

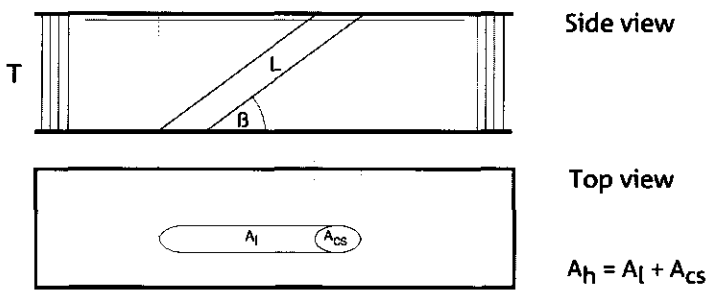

Figure 2.2. Schematic representation of a leaf tissue cross-section containing one hyphal element. The side view of the section shows a hyphal element of length L oriented in the section under angle β. T represents the section thickness. The lower figure represents the section as it is seen through the microscope with the projected surface area of the hyphal element. According to the principle of Delesse the surface area of the cross section through the hyphal element (A_{cs}) is the correct estimator for the relative volume of the hyphal element in the section. For very thin sections (section thickness: $T \approx 0$), the relative surface area of the hyphal element in the section is $A_h = A_{cs}$. The second line from the top (thin) in the side view represents the lower border of such a section. For thicker sections, A_h is composed of A_{cs} plus the relative surface area of the projection of the body of the hyphal element: $A_h = A_l + A_{cs}$. Thus in thicker sections, A_h overestimates the relative volume of the hyphal element in the section by the projected surface area of the body of the hyphae (A_l). Our calculation method for V_h using equations 2.1 and 2.2 corrects for this overestimation.

Mycelial Volume

According to the principle of Delesse (1847), the measured relative surface area or areal density of hyphal elements in sections (A_h) is a good estimator for the relative volume or volume density of the hyphal elements (V_h) provided that the section is very thin. However, in our relatively thick sections, A_h overestimates V_h due to a significant contribution of the projection of the hyphal element (A_i) to A_h (Fig. 2.2). The areal density of the *Botrytis* spp. in the section (A_b, unitless) is measured using the Bc-KH4 - FITC label after which equation 2.1 is used to calculate V_b, the unitless volume density of the *Botrytis* spp. The total areal density (A_h, unitless) is measured using the WGA-TRITC label. Substitution of V_b and A_h in equation 2.2 allows calculation of V_u (unitless), the volume density of *U. atrum*. Both conversion formulas are based on established stereological principles. The complete derivation of equations 2.1 and 2.2 is given in the appendix.

$$1 - A_b = (1 - V_b)\, e^{\left(-V_b \frac{T}{2r_b}\right)} \qquad \text{(equation 2.1)}$$

$$1 - A_h = (1 - V_b - V_u)\, e^{\left(-V_b \frac{T}{2r_b} - V_u \frac{T}{2r_u}\right)} \qquad \text{(equation 2.2)}$$

In equations 2.1 and 2.2, A_h and A_b represent the relative surface area of the section covered by the projection of hyphal elements of both species and the *Botrytis* spp. respectively while V_b and V_u represent the relative volume of the section occupied by hyphal elements of the *Botrytis* spp and *U. atrum*. T is the section thickness (µm) and r_b and r_u represent the hyphal radius (µm) of the *Botrytis* spp. and *U. atrum*. Thus, the relation between A_h and V_h is governed by the ratio between section thickness and the hyphal radii (Fig. 2.3). For known dimensions of the section, V_h can easily be converted into the absolute volume of the hyphal elements in the sections.

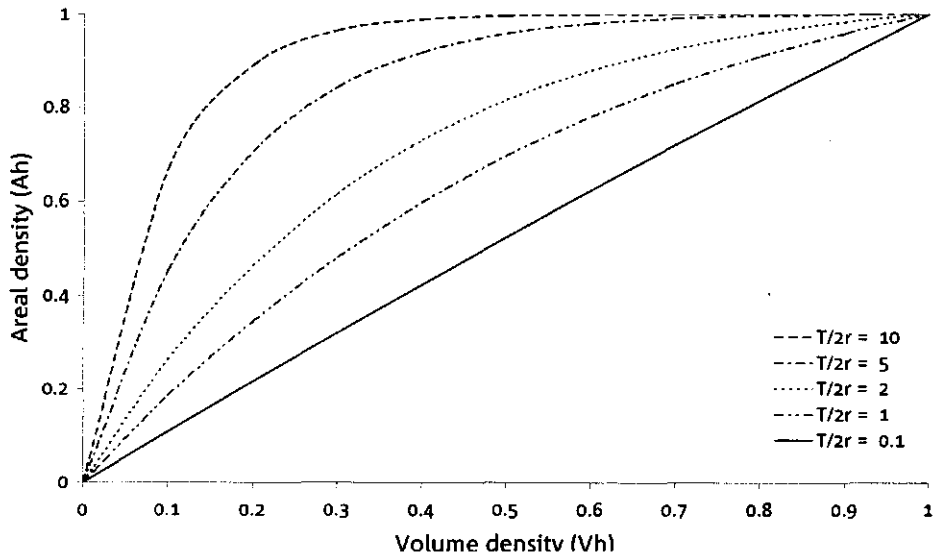

Figure 2.3. Theoretical relationship between the measured areal density (A_h) and the volume density (V_h) of hyphal elements in sections according to equation 2.1 for different values of the ratio between section thickness and radius of the hyphal elements (T/(2r)). Section thickness (T) and hyphal radius (r) determine the contribution of the projection of the body of the hyphal element to the measured areal density (A_h) plus the chance of hyphal elements overlapping in the image used to measure the surface density. The ratio (T/(2r)) therefore governs the theoretical relationship between the areal density (A_h) and the volume density (V_h). Volume densities of *U. atrum* and *B. cinerea* in Table 2.2 were calculated using a section thickness of 8 µm. Hyphal radii for *U. atrum* and *B. cinerea* were 2.1 and 2.6 µm respectively. This results in a T/(2r) ratio of approximately 2.

Detection of *Botrytis* spp. and *U. atrum* in tissue

Two experiments were carried out on sterile necrotic cyclamen leaves with *B. cinerea* and *U. atrum* and one on sterile necrotic lily leaves with *B. elliptica* and *U. atrum*. The first cyclamen experiment was used to obtain estimates for the variance components at the different sampling levels of leaf, leaf-sample, section within sample and position within section. Variance components are indicative of the variability of fungal colonization between and within leaves and can be used to design optimal sampling strategies. Five sterile necrotic cyclamen leaves were inoculated with a conidial suspension of *B. cinerea* and another five leaves were inoculated with a

conidial suspension of *U. atrum*. Petri dishes were incubated at 18°C in the dark in a completely randomized design. After 36 h of incubation, 4 samples approximately 3x5 mm in size were taken per leaf and prepared for sectioning. Per sample, two sets of approximately eight sections were collected, each set on a separate glass slide. The sets originated from locations 300 - 400 µm apart. Sections within a set were cut in sequence. Tissue sections were stained using the nonspecific fluorescent stain (WGA-TRITC) only. One randomly chosen section per set was analysed. Within sections three positions were distinguished, the centre of the section and two positions left and right from the centre near the edges of the section. Measurements were taken from three positions per sample including a randomly chosen position in a section from the first set and from the two complementary positions in a section from the second set of a sample. Including measurements on all three positions per section would have doubled the number of measurements without a significant effect on the precision of the estimates. This resulted in a total of 120 measurements (2 fungal species, 5 leaves per species, 4 samples per leaf and 3 positions per sample). For both fungi, the importance of variation between and within leaves as well as systematic differences between positions were examined by fitting a mixed model with position as the explanatory variable and random effects for differences between leaves, between samples within leaves and between sections within samples. In the model the random effects are assumed to be normally distributed with mean zero and variances σ^2_{leaf}, $\sigma^2_{samples\ within\ leaves}$ and $\sigma^2_{section\ within\ sample}$ respectively.
Estimates of fixed effects of position and variance components were obtained using the method of residual maximum likelihood (REML) in Genstat 5 (Numerical Algorithms Group, Oxford, UK). Systematic effects of position were assessed by the Wald statistic using the chi-square test.
The second cyclamen experiment was designed to monitor colonization levels of the pathogen and the antagonist in a short time series. Sterile necrotic cyclamen leaves were sprayed with conidial suspensions of *B. cinerea*, *U. atrum*, both fungi or sterile tap water + 0.01% Tween 80 and incubated at 18°C in the dark in a completely randomized design. Six replicate leaves per treatment and sampling time were included. Leaves were destructively sampled 0, 30 and 51 hours after spraying. In accordance with the results of the analysis of components of variance, colonization levels were determined for the centre position of one section of one sample for each leaf. Colonization data for *U. atrum* and *B. cinerea* were subjected to analysis of variance (ANOVA) testing for effects of fungal inoculum, sampling time and their interaction. In case *F*-tests were significant ($P < 0.05$), LSD tests ($P = 0.05$) were used for testing pairwise differences between the means.

In the lily experiment, sterile necrotic lily leaves were sprayed with conidial suspensions of *B. elliptica* and *U. atrum* or sterile tap water + 0.01 % Tween 80. *U. atrum* was applied 8 hours after *B. elliptica*. petri dishes were incubated at 18°C in the dark in a completely randomized design. Four replicate petri dishes with four leaves per dish were used per treatment. Colonization levels were determined 0, 30 and 51 hours after application of the *B. elliptica* inoculum.
The hyphal diameter was measured in WGA-TRITC labelled sections of both colonization experiments in sections sampled after 30 and 51 hours incubation. The hyphal radius, required for the conversion of areal densities of fungal colonization to the volume density (equations 2.1 and 2.2), was calculated as the average of 24 measurements per species *B. cinerea*, *B. elliptica* and *U. atrum* were found to have hyphal radii of 2.6 ± 0.3 , 2.7 ± 0.1 and 2.1 ± 0.3 µm respectively.

Results

Immunofluorescent labelling and general lectin stain

The staining procedure resulted in a bright and consistent staining of hyphae of *B. cinerea*, *B. elliptica* and *U. atrum* by the WGA-TRITC label (Fig. 2.1A) and of *B. cinerea* and *B. elliptica* hyphae by the MAb BC-KH4 + anti-mouse FITC conjugates (Fig. 2.1B). Hyphae of *Botrytis* spp. were visible with the TRITC- and the FITC-selective filter, whereas *U. atrum* hyphae were only visible with the TRITC-selective filter. Differences in staining intensity between *B. cinerea*, *B. elliptica* and *U. atrum* for the WGA-TRITC stain were not observed. FITC labelled hyphae in sections containing only *U. atrum* were never observed. Green or red fluorescing structures in sections of non-inoculated leaves were not observed. This made it possible to distinguish the fungi from the background and to distinguish between the mycelia of the respective pathogens and the antagonist.

Detection threshold, quenching and reproducibility

The detection program was applied on digitized images obtained from the CCD camera. Structures represented by one pixel were the smallest structures that could be detected. This corresponded to structures 1.2 x 1.2 µm in size at a 200x magnification. Smaller structures could be detected using higher magnifications.
Similar to other fluorescent stains the intensity of the stains described here was subjected to quenching. However, with our staining procedure the quenching rates were relatively low when compared to the quenching rate of fluorescein diacetate (FDA). When the measurements on the section in Fig. 2.1. were repeated seven months after the original measurements, during which time the sections were stored at 4°C in the dark, the relative surface areas detected were 4% for *B. elliptica* and 14% for *U. atrum* as compared to 5% and 17% respectively in the original measurements. When the section was exposed to continuous illumination using the HQ-filter block for TRITC detection, the detected total surface area covered by fungal hyphae was reduced by 7% in 15 minutes and by 53% in 60 minutes (data not shown). This low quenching rate allowed ample time for measurements, which took about 1 minute per sample. These results also indicate that it is possible to store non exposed sections for at least several months at 4°C in the dark without significant loss of quality.
Reproducibility was tested by repeated measurements on the same section. For this purpose a section of lily tissue colonized by *B. elliptica* and *U. atrum* after 51 hours of incubation was used. Four replicate measurements resulted in almost identical results: 6.5 ± 0.3% colonization by *B. elliptica* and 7.2% ± 0.3% colonization by *U. atrum*. Small differences in the four images obtained from the section by the integrating CCD camera were the most likely cause for the differences that were found.

Variance components

Variance components for the different sampling levels of *B. cinerea* or *U. atrum* colonized necrotic cyclamen leaves are given in Table 2.1. The calculated *P*-value of the chi-square test for the Wald statistic ($P = 0.064$), indicated that measurements near the edges of a section tend to be lower than measurements near the centre of the section. This clear edge effect may be the result of over fixation of the exposed edges of a tissue sample by the paraformaldehyde which is capable of blocking the

TABLE 2.1. Components of variance with standard errors in parenthesis indicative of variability of fungal colonization between and within leaves.

Source of variation[a]	*Ulocladium atrum*	*Botrytis cinerea*
Leaves (σ^2_{leaf})	5.5 (4.7)	5.6 (4.8)
Samples within leaves (σ^2_{sample})	2.8 (1.9)	0.7 (2.4)
Sections within samples ($\sigma^2_{section}$)	0.9 (1.7)	1.3 (3.2)
Position within sections ($\sigma^2_{position}$)	3.8 (1.2)	8.3 (2.7)

[a] The importance of variation between and within leaves as well as systematic differences between positions were examined by fitting a mixed model with position as explanatory variable and random effects for differences between leaves, between samples within leaves and between sections within samples. Estimates of fixed effects of position and variance components were obtained using the method of residual maximum likelihood (REML) in Genstat 5. Systematic effects of position were assessed by the Wald statistic using the chi-square test.

binding sites for the MAb and the lectin through extensive cross-linking. This problem can be avoided by measuring at less exposed locations near the centre of the section.

For both fungi, the estimated components of variance in Table 2.1 suggest a large variation in colonization levels between leaves. The estimated components of variance from sources within the leaf suggest a spatially inhomogeneous colonization of leaves. Residual error variances generally were large but because they are asymptotically estimated their informative value is limited.

Based on the size of the estimated components of variance from the present experiment, the expected variance of the mean colonization level of a new experimental design can be calculated according to:

$$var_{mean} = (\sigma^2_{leaf}/n_{leaf})*\{1+1/n_{sample} * \{\sigma^2_{sample}/\sigma^2_{leaf} + (\sigma^2_{section}/\sigma^2_{leaf})/n_{section} + (\sigma^2_{position}/\sigma^2_{leaf})/(n_{section}*n_{position})\}\}$$

Costs of labour and materials for the different steps in the sampling, processing and measurement can be taken into account by introducing cost functions (Sokal and Rohlf, 1995). Thus, the most efficient sampling scheme for each purpose can be designed. From the estimated components of variance it is concluded that, in general, sampling schemes will be most efficient when the number of leaves is maximized with as little effort as possible at the lower sampling levels.

TABLE 2.2. Effect of fungal inoculation (Treatment) and incubation period (Time) on the volume density of *Ulocladium atrum* and *Botrytis cinerea*, representing fungal colonization, in sterile necrotic cyclamen leaf tissue.

ANOVA[a]			*F* - probability	
Effect			*Botrytis cinerea*	*Ulocladium atrum*
Treatment			0.109	0.027
Time			0.098	0.004
Treatment * Time			0.150	0.033
Comparison of means			Average volume density (%)	
			Botrytis cinerea	*Ulocladium atrum*
Treatment	*B. cinerea*		0.7	0[c]
	U. atrum		0[b]	1.6
	B. cinerea + *U. atrum*		0.3	0.4
Time	0 hours		0[b]	0[b]
	30 hours		0.3	0.2
	51 hours		0.7	1.8
Treatment * Time	*B. cinerea*	0 hours	0[b]	0[b]
		30 hours	0.3	0[c]
		51 hours	1.2	0[c]
	U. atrum	0 hours	0[b]	0[b]
		30 hours	0[b]	0.3
		51 hours	0[b]	2.9
	B. cinerea + *U. atrum*	0 hours	0[b]	0[b]
		30 hours	0.2	0.2
		51 hours	0.3	0.7
	$LSD_{0.05}$			1.4

Sterile necrotic cyclamen leaf tissue was homogeneously inoculated with conidial suspensions of *B. cinerea*, *U. atrum* or a combination of both. Conidial concentrations were $1x10^5$ conidia/ml for both species, resulting in conidial densities of 26 ± 8 and 23 ± 11 conidia/mm^{2}leaf for *U. atrum* and *B. cinerea* respectively. Inoculated tissue was destructively sampled after incubation in moist chambers at 18°C in the dark. Average volume densities (mycelial volume / volume of section (%)) are based on one measurement in one section in each of six replicate leaves except for the *U. atrum* volume density measured after 30 hours incubation in leaves inoculated with *U. atrum* only which is based on one measurement in one section in each of four replicate leaves. The volume density was calculated from the measured areal density using equation 2.1 and 2.2. Section thickness was 8 µm, hyphal radii for *U. atrum* and *B. cinerea* were 2.1 and 2.6 µm respectively.

[a]: Analysis of variance (ANOVA) and comparison of means on volume densities per leaf.

[b]: Excluded in the analysis of variance due to absence of variance in the observed data.

[c]: Excluded because of negative values. *B. cinerea* mycelium was quantified using the FITC label and the TRITC label. Small differences between the images from both labels result in detection of small positive or negative quantities of *U. atrum* mycelium. Standard deviations were 0.2 and 0.3% after 30 and 51 hours of incubation respectively.

Temporal aspects of substrate colonization

As indicated by the significant effect of time on the *U. atrum* colonization level and the indication of significance for *B. cinerea* (Table 2.2) the volume density of both fungi in cyclamen tissue increased during the entire incubation period. When *B. cinerea* or *U. atrum* were applied alone, the average volume density increased from 0% to 0.3% and 1.2% for *B. cinerea* and from 0% to 0.3% and 2.9% for *U. atrum* after 0, 30 and 51 hours of incubation, respectively. When both fungi were applied in combination, both actively colonized the available tissue. The colonization levels reached after 51 hours of competitive colonization were however lower then the levels reached during non competitive colonization (Table 2.2). This trend was only apparent after 51 hours incubation accounting for the significant interaction between the effects of time and treatment on the *U. atrum* volume density. Apparently *U. atrum* and *B. cinerea* are only hindering each other after the first phase of colonization. No fungal colonization was measured in leaf tissue sprayed with water containing 0.01% Tween 80 only (data not shown).
In comparison, the volume densities reached in sterile necrotic lily tissue by *B. elliptica* and *U. atrum*, applied in combination, increased from 0% to 0.4% and 1.3% for *B. elliptica* and from 0% to 0.8% and 3.1% for *U. atrum* after 0, 30 and 51 hours of incubation, respectively. Similar to the situation in cyclamen, the pathogen and the antagonist colonized the available tissue in each others presence. Necrotic lily leaf tissue however, supported higher fungal colonization levels than necrotic cyclamen leaf tissue.

Discussion

A technique was developed to localize and quantify the mycelium of *Botrytis* spp. and *Ulocladium atrum* within necrotic tissue. It provides both qualitative (visual) and quantitative information on the internal component of the fungal colonization of necrotic plant tissue.

The combination of a specific immunofluorescent label and a lectin conjugated to a fluorochrome is equivalent to the method reported by Pfender et al. (1991) who used a dual-staining technique to observe antagonism of *Pyrenophora tritici-repentis* by the antagonistic fungus *Limonomyces roseipellis* in wheat straw. In their situation however, quantification of mycelium was not possible and the selectively stained fungus was not always visible with the non-selective stain. The latter difference might be caused by a difference in affinity for WGA-TRITC between the fungi used in both studies.

To obtain an accurate estimate for the fungal colonization of the tissue section it is necessary that the whole hypha is stained. In our case, both the chitin residues and the antigen to which the MAb BC-KH4 binds are located throughout the fungal cell wall (Cole et al., 1996), ensuring that both stains visualize the full hyphae.

A common problem encountered when visualizing fungal hyphae within plant tissue using fluorescence microscopy is host autofluorescence, obscuring the fluorescence of the stained fungal hyphae. Several techniques have been applied to reduce host autofluorescence including enzymatic degradation of stained host cellulose (Daniel et al., 1995; Trese and Loschke, 1990) and counterstains (Pfender et al., 1991). During the present study, the extent of the autofluorescence problems was largely determined by the host species. Autofluorescence problems were minimized by the use of a specific MAb which did not cross-react with host tissues, a narrow band filter for the FITC detection and the shading correction. For cyclamen tissue, it also was necessary to use acetylated BSA (BSA-C) and normal goat serum. BSA-C is effectively suppressing charge determined background fluorescence (van de Plas and Leunissen, 1993) whereas normal goat serum prevents aspecific binding of the (goat-anti mouse) FITC-conjugates. Autofluorescence countermeasures as applied by Pfender et al. (1991), Trese & Loschke (1990) or Daniel et al. (1995) were not necessary.

For image analysis it is necessary to separate the target structures from the rest of the image. This separation can be based on differences in grey value between the target and the background and/or structural characteristics of the target (e.g. length, width, shape). Overlapping hyphae, cut at different angles, result in a wide variety

of possible shapes of fungal mycelium in the section. This makes it impossible to separate hyphae from the background using structural characteristics. The detection was therefore based on differences in grey value between the fluorescing hyphae and the non-fluorescent background. Epifluorescence microscopy is especially suited for this purpose because it usually results in high contrasts. The higher the contrast between the hyphae and the background the more accurate the detection will be. The use of the integrating CCD camera and the image correction through the subtraction of a background image from the original grey value FITC and TRITC images greatly enhanced the contrast between target structures and non-target structures. However, weakly fluorescing hyphal structures might not be detected using this procedure. Our method is also sensitive to differences in staining intensity of the fungal hyphae within experiments and between experiments. The background correction of the grey value images and the manual adjustment of the detection threshold between experiments are tools used to overcome this problem. Nevertheless, the stains applied have to produce high contrast images for the method to produce accurate results.

The *B. cinerea* areal density can be measured with both the general WGA-TRITC label and the specific Bc-KH4-FITC label. A measurement on the same section with each of the two labels will result in two nearly identical values for the *B. cinerea* areal density. Small differences between the measured values for each of the labels however, result in small quantities of *U. atrum* detected because *U. atrum* colonization is calculated as the difference between the surface area detected with the WGA-TRITC label and the surface area detected with the Bc-KH4 label. Although the average volume density of *U. atrum* detected in tissue inoculated with *B. cinerea* only is zero for all three sampling times, the bias in the detection of *U. atrum* is evident through the standard deviations of 0.2% and 0.3% after 30 and 51 hours of incubation (Table 2.2). This problem is not relevant in situations where it is known that only *B. cinerea* is present but it influences the sensitivity of the detection of *U. atrum* in situations where both *U. atrum* and *B. cinerea* are present. The problem can be solved by using a specific label for both fungal species making the indirect detection of one of the species obsolete.

The principle of Delesse (1847), applied on leaf tissue sections colonized by fungi, states that the areal density of the cross sections through the hyphae (A_{cs} in Fig. 2.2) is a good estimator for the volume density of the hyphal elements in the section. The areal density as measured using our technique would therefore be a good estimator for the volume density for very thin sections (Fig. 2.2). For thicker sections, equations 2.1 and 2.2 allow one to correctly estimate the volume density which, in contrast to

the areal density, is independent of section thickness. These equations are based on the principle of Delesse (1847) and the basic theories used to calculate the projected leaf area of crop canopies (Goudriaan and van Laar, 1994). The volume of hyphal elements in a section is an excellent estimator for fungal biomass. On top of this, the conversion to volume allows a direct comparison of results obtained using sections of different thickness. This conversion therefore adds in the flexibility of this technique and facilitates a more accurate analysis of the generated data. The low values found for the areal densities of the fungi in the sections belong to the first near-linear part of the curves in Figure 2.3, indicating that overlap in the projection of the hyphae hardly occurs.

At present, a direct comparison between our technique and chemical, immunological or molecular techniques is not possible because the other techniques do not distinguish between fungal species. A comparison with only one species would be inconclusive because our technique quantifies the internal component of fungal colonization whereas the other techniques quantify total colonization: internal plus external mycelium, conidiophores and conidia. The dynamics of the internal and external colonization are likely to be different. The internal, actively competing, mycelial component of fungal colonization is our prime interest when studying competitive substrate colonization by *U. atrum* and *Botrytis* spp.

Measurements on the colonization process of necrotic cyclamen and lily tissue by *Botrytis* spp. and *U. atrum* show that the species are capable of co-colonizing these tissues. However, growth tends to be reduced when both species are present as compared to when the species are alone, especially during the second sampling interval. This observation, together with the observations by Köhl et al. (1997) indicating that toxins are not involved in the interaction between *Botrytis* spp. and *U. atrum,* supports the hypothesis of nutrient competition as the mode of action. The analysis of variance components reveals that despite homogenous inoculation, resulting in high and homogenous initial conidial densities on the leaf surfaces, the resulting colonization of necrotic tissue is not homogenous. Large differences are found in the colonization levels between leaves and within leaves (Table 2.1). We believe that this variation reflects qualitative structural and nutritional differences between leaves and within leaves with respect to colonization by *B. cinerea* and *U. atrum*.

A disadvantage of our technique is that it cannot distinguish between viable and non-viable mycelium. This disadvantage is shared with most other techniques quantifying fungal colonization. This fact has to be included in the interpretation of experimental results, especially for the later phases of competitive substrate colonization.

The technique described in this paper quantifies the internal fungal colonization of necrotic tissue as a measure for the fungal biomass present internally in the tissue. It is capable of distinguishing between fungal species and can be applied in at least two host plants. The relatively simple principles underlying the quantification of fungal colonization as well as the basic nature of the image analysis tools used facilitate successful adaptation to other systems and environments. At present, the availability of specific fungal labels seems to be the most limiting factor for a wider application. If specific antibodies for each of the two fungi studied were conjugated directly, each to a different fluorochrome, our technique could also be applied to field situations to quantify the mycelia of the target fungi in the presence of naturally occurring saprophytic fungal species.

Our technique generates a unique combination of quantitative and qualitative data on competitive substrate colonization, a key process in the biological control of *Botrytis* spp. by *U. atrum*. It is even sensitive enough to provide information on the potentially decisive early stages of colonization. We therefore conclude that it has the potential to provide new insights into fungal ecology essential for the development of new fungal biocontrol agents.

Appendix

To determine the volume density of hyphal elements in a leaf section, a stereological conversion formula was derived. The areal density of the hyphal elements in the section (A_h; the relative surface area of the projection of the hyphal elements (Weibel, 1979)) is measured using image analysis techniques. The volume density (V_h; the relative volume of the hyphal elements in the section (Weibel, 1979)), would however be a better estimator for the fungal biomass present in the section. According to the principle of Delesse (1847), for very thin sections $A_h = V_h$.
In very thin sections A_h is only constituted by A_{cs}, the relative surface area of the cross sections through the hyphae (Fig. 2.2). In thicker sections however, A_h is composed of two components: A_{cs} plus the projection of the body of the hyphal element in the section (A_l):

$$A_h = A_l + A_{cs} \qquad \text{(equation 2.1A)}$$

Thus, A_{cs} is the correct estimator for V_h. In thicker sections, the measured, A_h overestimates V_h increasingly with an increasing section thickness.
To allow an accurate estimate of V_h, a mathematical expression for A_l is derived and used to calculate A_{cs}, as an estimator for V_h, based on (2.1A).
Hyphae are considered as cylindrical elements, placed within the volume of a section. For each species, the separate elements are assumed to have an identical radius (r) and to be oriented in all possible directions. The measured absolute area of the section is A, the calculated relative surface area of the hyphal elements in the section is A_h and the known thickness of the section is T. The, unknown, total length of the hyphal elements in the section is L. The absolute volume of the section (V) can be calculated as: V = AT.
The true relative surface area of hyphal elements, in horizontal orientation and without overlap, would be: 2rL/A.
Analogous to the calculation of the projected area of several leaf layers of a crop canopy (Goudriaan and van Laar, 1994), the relation between the measured relative surface area of the hyphal elements and the true relative surface area of the hyphal elements is given by:

$$A_l = 1 - e^{\left(-k \cdot \frac{2 \cdot r \cdot L}{A}\right)} \qquad \text{(equation 2.2A)}$$

The proportionality factor k compensates for the different orientations of hyphal fragments whereas the exponential function corrects for hyphae overlapping in projection. Assuming a spherical (or isotropical) distribution of the angle of the hyphal fragments with the horizon (β in Fig. 2.2), k can be calculated as the ratio between the projected surface area of the hyphal fragments in a spherical distribution (without overlap) and the true surface area of the hyphal fragments (Goudriaan and van Laar, 1994): The projected surface area of hyphal elements, oriented in the section under an angle β, is calculated as 2rLcos(β) with a frequency distribution of β that, in a spherical distribution, follows that of cos(β). The true surface area of the hyphal fragments is 2rL. Therefore:

$$k = \frac{1}{2rL} \int_{0}^{\frac{1}{2}\pi} 2rL \cos^2(\beta)\, d(\beta) = \frac{\frac{1}{2}\pi rL}{2rL} = \frac{1}{4}\pi \qquad \text{(equation 2.3A)}$$

Equation (2.2A) combined with the result of (2.3A) gives the total relative projection of hyphal fragments with a spherical distribution:

$$A_l = 1 - e^{\left(\frac{-\pi r L}{2A}\right)} \qquad \text{(equation 2.4A)}$$

The relative volume of the hyphal elements in the section can be calculated as:

$$V_h = \frac{\pi r^2 L}{A T} \qquad \text{(equation 2.5A)}$$

Equations (2.4A) and (2.5A) can be combined into:

$$A_l = 1 - e^{\left(V_h \frac{T}{2r}\right)} \qquad \text{(equation 2.6A)}$$

A_l will only contribute to A_h when the projection of the body of the hyphal element is not overlapping with a cross-section through a hyphal element (A_{cs}). A_l will therefore only contribute to A_h for a fraction ($1-A_{cs}$) = ($1-V_h$) of the available surface A. Thus (2.6A) becomes:

$$A_l = (1 - V_h) \left(1 - e^{\left(-V_h \frac{T}{2r} \right)} \right)$$ (equation 2.7A)

When we substitute (2.7A) into (2.1A) and substitute A_{cs} by V_h we get the desired relationship between the measured areal density (A_h) of hyphal elements in a cross-section and the volume density:

$$1 - A_h = (1 - V_h) e^{\left(-V_h \frac{T}{2r} \right)}$$ (equation 2.8A)

In (2.8A) $1-A_h$ represents the relative surface area of the section not covered by the cross-sections through fungal elements or projection of fungal elements whereas $1-V_h$ represents the non-fungal relative volume in the section. V_h cannot be written explicitly from (2.8A) but it can readily be solved numerically. Thus, the relation between the measured surface density (A_h) and the hyphal volume density is governed by the ratio between the section thickness (T) and the hyphal diameter (2r) of the fungal species under consideration (Fig. 2.3). Since r can be measured and T is known, the absolute hyphal volume in the section can also be calculated.
When both, *Botrytis* spp. and *U. atrum* are present in the section, the total volume density (V_h) is equal to the volume density of the *Botrytis* spp. (V_b) plus the volume density of *U. atrum* (V_u):

$$V_h = V_b + V_u$$ (equation 2.9A)

The areal density of the *Botrytis* spp. in the section (A_b) is measured specifically using the Bc-KH4-FITC label. In this case $A_h = A_b$ and (2.8A) can be converted into (2.10A) which is used to calculate the volume density of the *Botrytis* spp in the section (V_b). r_b represents the hyphal radius of the *Botrytis* spp.

$$1 - A_b = (1 - V_b) e^{\left(-V_b \frac{T}{2r_b} \right)}$$ (equation 2.10A)

For two fungal species (2.10A) can be extended to (Combine (2.8A) and (2.9A)):

$$1 - A_h = (1 - V_b - V_u) e^{\left(-V_b \frac{T}{2r_b} - V_u \frac{T}{2r_u} \right)}$$ (equation 2.11A)

A_h now represents the total areal density as it is measured using the WGA-TRITC label, r_b and r_u represent the respective hyphal radii of the *Botrytis* spp. and *U. atrum*. V_b is calculated directly using (2.10A) and substituted in (2.11A) to calculate V_u.

Chapter 3

Competitive substrate colonization by *Botrytis cinerea* and *Ulocladium atrum* in relation to biological control of *Botrytis cinerea* in cyclamen

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To be submitted

Abstract

The temporal dynamics of colonization of necrotic cyclamen leaf tissue by *Botrytis cinerea* and *Ulocladium atrum* were studied. The development of mycelial biomass and sporulation in time followed logistical patterns for both species in monocultures and in mixed cultures. The maximum colonization and sporulation levels reached by the individual fungi were lower if they were competing for the same substrate. The carrying capacity for fungal mycelium varied substantially between individual leaves and batches of leaves. The level of sporulation was much less influenced by leaf quality. The hypothesis of nutrient competition as the dominant antagonistic mechanism between *B. cinerea* and *U. atrum* was confirmed in histological studies, by 'de Wit' replacement series, by pairwise confrontations between mycelia in necrotic cyclamen leaf tissue and by correspondence of experimental results with results of a dynamic simulation model based on nutrient competition. Using de Wit replacement series, the spectrum of resources in necrotic cyclamen leaves available to *U. atrum* was shown to include the whole spectrum of essential resources available to *B. cinerea*. A small amount of resources in the necrotic cyclamen leaves could exclusively be assimilated by *U. atrum*. Competition between the mycelia of *B. cinerea* and *U. atrum* reduced the sporulation of both species but the reduction was smaller than expected on the basis of equal inter- and intraspecific competition. The results show that *B. cinerea* can be completely excluded from necrotic cyclamen leaves as a result of pre-emptive resource capture by *U. atrum*, provided the antagonist is given early enough access to the substrate. *B. cinerea* could never completely exclude *U. atrum*. This principle may be employed to achieve biological control of *B. cinerea* and possibly other necrotrophic fungi.

Introduction

Botrytis cinerea Pers. ex Pers. is the cause of economically important diseases in many field and greenhouse crops. In commercial cyclamen (*Cyclamen persicum* L.) crops, *B. cinerea* is considered a major pathogen causing leaf rot (Nightingale, 1982; Jacob, 1987). As a necrotroph *B. cinerea* kills healthy plant tissue before colonizing it or it saprophytically colonizes already necrotic tissue. In cyclamen, *B. cinerea* conidia initiate saprophytic colonization of necrotic tissue before mycelium of *B. cinerea* is capable of attacking and infecting neighbouring healthy petioles and leaf blades (Chapter 4). *B. cinerea* conidia are not capable of infecting healthy cyclamen petioles or leaves directly (Schlösser, 1978).
Ulocladium atrum Preuss, isolate 385, is a saprophytical antagonist of *Botrytis* spp. effectively suppressing the pathogens sporulation in necrotic plant tissue (Köhl et al.,

1995b). In commercial cyclamen crops *U. atrum* applications were as effective against *B. cinerea* as the growers standard fungicide program (Köhl et al., 1998). The control effect of *U. atrum* against *Botrytis* spp. is based on both species competitively interacting in necrotic plant tissue (Köhl et al., 1997; Chapter 4).

A thorough knowledge of competitive substrate colonization by *B. cinerea* and *U. atrum* is essential to identify and understand the possibilities and limitations of the antagonist and identify options to manipulate the interaction in favour of the antagonist. At present, knowledge of the competitive interaction between *B. cinerea* and *U. atrum* is limited and the mechanism through which they are competing is not unequivocally established. Microscopic and ultra structural studies (Köhl et al., 1997) suggest that toxins and direct parasitism are not involved. Competition for nutrients is therefore believed to be the dominant mechanism with the mycelium growing internally in the tissue as the most actively competing component of fungal colonization. Internal mycelium exploits available resources and as opposed to external components of colonization is protected from adverse conditions in the phyllosphere. Internal mycelium is therefore crucial for an efficient exploitation of the available resources and for survival and sporulation of fungi.

Studies on competitive interactions between fungi often rely on quantification of sporulation (Biles and Hill, 1988, Elad et al., 1994a, Köhl et al., 1995b,) or fruiting bodies (Adee et al., 1990, Newton et al., 1997). These techniques do however not give information about the potentially decisive early phases of colonization or the extent of internal colonization of a substrate.

This paper aims to elucidate mechanistic principles behind the competitive interaction between *B. cinerea* and *U. atrum* in necrotic cyclamen leaf tissue. Substrate colonization was studied after homogeneous- or after point inoculation of necrotic leaves. Internal mycelial colonization and/or sporulation were quantified in time series or after two weeks incubation. Results from the different experiments were integrated in a dynamic simulation model to check the validity of the hypothesis formulated with respect to the mechanistic principles behind competitive substrate colonization by *B. cinerea* and *U. atrum*.

At the macroscopical leaf level, the competitive interaction between *B. cinerea* and *U. atrum* was studied after homogeneous inoculation using a technique similar to a de Wit replacement series (de Wit, 1960). Plant ecologists used this technique to study competitive interactions between herbaceous plant species (e.g. de Wit et al., 1966). Mycologists used it to study the interaction between competing fungi e.g. *Pyrenophora tritici-repentis* and *Septoria nodorum* in wheat leaves (Adee et al., 1990) and *Ceratocystiopsis ranaculosus*, *Entomocorticium* spp. and *Ophiostoma minus* on

the southern pine beetle (*Dendroctonus frontalis*) (Klepzig and Wilkens, 1997). In replacement series studies, the proportions of two (micro-) organisms grown in mixture are varied while the total inoculum density is held constant. In our studies, we kept the inoculum densities of the two competing species constant over all treatments, but varied the time of inoculation, giving one of the species a head start. The analysis proceeds in a similar fashion as de Wit's (1960). A key concept is the relative yield (RY, yield in mixture divided by the yield in monoculture) for both competing species and the relative yield total (RYT, the sum of the relative yield for both species). The behaviour of relative yields in response to treatments provides evidence for the nature and strength of interactions between species (Braakhekke, 1980).

Temporal aspects of substrate colonization and competition were studied using a combination of microscopic and macroscopic methods. Following homogeneous inoculation, internal mycelial colonization at tissue level and the resulting sporulation, observed at leaf level, were quantified. For this purpose differential fluorescent labelling of the mycelia (Chapter 2) was combined with visual assessment of sporulation (Köhl et al., 1998). Supplementary to the homogeneously inoculated experiment, the interaction between mycelia of *B. cinerea* and *U. atrum* was also studied using a confrontational approach with pairwise point inoculations of necrotic cyclamen leaves.

Finally, experimental results were compared to the results of a dynamic simulation model. The model originated from a more theoretical approach and simulated growth of biomass of *B. cinerea* and *U. atrum* in necrotic tissue based on the hypothesis of nutrient competition as the only antagonistic mechanism. The comparison between model results and experimental results was used to evaluate the hypothesis of nutrient competition as the prevailing antagonistic mechanism between *B. cinerea* and *U. atrum*.

Materials and Methods

Cultures

Botrytis cinerea, isolate 700, originally isolated from gerbera was stored as a conidial suspension in sterile tap water containing 0.01% Tween 80 (v/v) at -80°C. *Ulocladium*

atrum, isolate 385, originally isolated from a necrotic leaf tip of a field grown onion (Köhl et al., 1995b), was maintained on oatmeal agar (20 g of milled oat, 15 g of agar and 1 L of tap water) slants at 4°C in the dark. Before use, *B. cinerea* and *U. atrum* were cultured on oatmeal agar at 20°C in the dark for 2 weeks and 4 weeks, respectively. Conidial suspensions of both fungi were prepared by flooding the cultures with sterile tap water containing 0.01% Tween 80 (v/v). After gently rubbing with a rubber spatula to detach the conidia, suspensions were filtered through a nylon gauze with a mesh of 200 µm. Concentrations of the suspensions were determined using a haemocytometer and adjusted to the desired concentration with sterile tap water containing 0.01% Tween 80.

Plant material

Thirty week old cyclamen plants cv Super Serie were obtained from a commercial grower. The plants were placed on tables with capillary matting in a greenhouse set at 18°C. Relative humidity of the air (RH) varied between 80% and 90%. Daylight was supplemented using artificial lighting to a total of 10 hours of light per day. Leaves and flowers were removed and the plants were allowed to develop new pesticide and pollen free leaves. Pollen is known to enhance infection by *B. cinerea* (Chou and Preece, 1968). Plants were not treated with pesticides after they were defoliated. Flower buds were removed regularly. Three batches of fully grown symptomless leaves were picked in December, February and April. Leaves were placed on dry filter paper at room temperature and about 60% RH in a single layer and allowed to senesce. After two weeks, the leaves were dead. They were cut in half, the midvein was removed and the leaf halves were sealed in plastic bags and sterilised by gamma irradiation (4 Mrad). These half leaves are further referred to as leaves.

Head start experiments

Sterile necrotic cyclamen leaves were colonized by monocultures or mixed cultures of *B. cinerea* and *U. atrum* after homogeneous inoculation with conidia ($1x10^6$ conidial/ml). In the mixed cultures, the application interval between the two species was varied. Thus a range of different biomass ratios for *U. atrum* and *B. cinerea* at the moment of confrontation was created while the total inoculum density was kept constant. For the monocultures, identical application intervals were used but the

competitor was replaced by an application of sterile tap water containing 0.01% Tween 80.
Sterile necrotic cyclamen leaves were re-hydrated overnight in sterile tap water at 4°C. Excess water was removed by blotting the leaves on sterile filter paper and the leaves were placed in petri dishes (90 mm in diameter, four leaves per dish) on top of a sterile plastic grid which was placed on two sterile filter papers (80 mm in diameter) moistened with 1.5 ml of sterile tap water. Leaves in the petri dishes were homogeneously spray inoculated using atomizers (Desaga, Heidelberg, Germany) on the adaxial side of the leaves. Inoculation resulted in average conidial densities of 45 conidia/mm^2 leaf for *B. cinerea* and *U. atrum*.
Mixed cultures were initiated by spraying the leaves with conidial suspensions of *B. cinerea* and *U. atrum*. *B. cinerea* was applied 24, 12 and 0 hours ahead of *U. atrum* and 12, 24, 30, 36, 42, 48 and 72 hours after *U. atrum* had been applied.
Monocultures served as control treatments. They were initiated by spraying the leaves with a conidial suspension of *B. cinerea* or *U. atrum*. Sterile tap water containing 0.01% Tween 80 was sprayed on the leaves 24, 12 and 0 hours ahead of the conidial suspension and 12, 24, 30, 36, 42, 48 and 72 hours after the conidial suspension of *U. atrum*. For *B. cinerea*, sterile tap water containing 0.01% Tween 80 was applied 12, 24, 30, 36, 42, 48 and 72 hours ahead of the conidial suspension and 24, 12 and 0 hours after the conidial suspension. Petri dishes were incubated at 18°C in the dark for two weeks counted from the first spray.

Experimental design

Two replicate experiments were carried out, each with three spray treatments and ten application intervals. Five replicate petri dishes, with four leaves per dish, were included for each combination of spray treatment and application interval. Petri dishes were incubated in a completely randomized design.

Observations

Sporulation of *B. cinerea* and *U. atrum* was quantified two weeks after the first spray treatment. The fraction of the leaf area covered with conidiophores (0, 10, 20,...100%) and the relative intensity of the sporulation (0, 10, 20,...100%) of *B. cinerea* and *U. atrum* was recorded per leaf using a dissecting microscope at 100x magnification.

Multiplication of the two parameters and scaling of the result yields the Sporulating Leaf Area Corrected for Intensity (SPLACI, Köhl et al., 1998), an estimate for the number of conidia produced per leaf ranging from 0 - 100%. SPLACI values were averaged for the four leaves per petri dish. Sporulation was considered to be the yield of the fungi. Based on average SPLACI values per petri dish, the relative yields (RY, yield in mixed culture divided by the yield of the corresponding monoculture) of *B. cinerea* (RY_{bc}) and *U. atrum* (RY_{ua}) as well as their sum, the relative yield total ($RYT = RY_{bc} + RY_{ua}$), were calculated and plotted against the application interval on the x-axis. If inter- and intraspecific competition are equal in intensity, the RY of each species is directly proportional to its biomass proportion in the starting mixture. The sum of the relative yield for both species gives the relative yield total (RYT). The shape of the RYT line is indicative for the type of interaction between both competing species. A horizontal RYT line remaining at 1.0 for all input proportions indicates equal inter- and intraspecific competition. A RYT line that is concave or convex respectively indicate stronger and weaker interspecific than intraspecific competition.

Dynamics of mycelial colonization and sporulation

Internal mycelial colonization of sterile necrotic cyclamen leaves and the resulting sporulation in monocultures and in mixed cultures were monitored following homogeneous inoculation with conidia. Sterile necrotic cyclamen leaves, prepared as described before, were placed on top of a sterile plastic grid which was placed on top of two sterile filter papers (40 mm in diameter) moistened with 0.5 ml of sterile tap water in a sterile plastic petri dish (55 mm in diameter, one leaf per dish). Leaves were homogeneously spray inoculated on the adaxial side of the leaves with conidial suspensions ($1x10^6$ conidia/ml) of *B. cinerea*, *U. atrum* or both fungi, using an atomizer (Desaga, Heidelberg, Germany). Inoculation resulted in average conidial densities of 25 conidia/mm^2 for *U. atrum* and *B. cinerea*. Following inoculation, petri dishes were incubated in the dark at 18°C or 24°C.

Experimental design

Development of mycelium within the necrotic leaf material in time was studied in four experiments. *B. cinerea* and *U. atrum* were applied alone (all experiments) and

Table 3.1. Setup of four experiments on the dynamics of internal mycelium and sporulation of *Botrytis cinerea* and *Ulocladium atrum* in sterile necrotic cyclamen leaves.

Experiment	Treatment	Incubation temperature	Replicate leaves	Leaf batch	Sampling times (Hours after inoculation)
1	*B. cinerea*	18°C	5	1	0, 12, 24, 36, 48, 60, 72, 96, 120, 168, 212
	U. atrum				
2	*B. cinerea*	24°C	5	1	0, 12, 24, 36, 48, 60, 72, 96, 120, 168, 212
	U. atrum				
3	*U. atrum*	18°C	6	2	0, 36, 60, 96, 120, 168, 212, 260
	U. atrum	24°C	6		0, 36, 72, 120, 212, 260
4	*B. cinerea*	18°C	6	3	0, 30, 51, 100, 168, 216
	U. atrum				
	B. cinerea + *U. atrum*				

also in combination (experiment 4). Two incubation temperatures were included in the experiments (18°C and 24°C). Replicate leaves were included for each combination of inoculants, temperature and sampling time. Details on treatments, temperatures, leaf batch, replicates and sampling times for each of the experiments are given in Table 3.1. During the experiments, petri dishes were incubated in a completely randomized design.

Observations

Sporulation was quantified at each sampling time as the Sporulating Leaf Area Corrected for Intensity (SPLACI), as described for the head start experiments. Directly after sporulation had been quantified, leaf samples were taken, processed, sectioned on a cryostat and labelled as described in Chapter 2. Mycelium of *B. cinerea* in the tissue sections was labelled specifically using the IgM monoclonal antibody (MAb) Bc-KH4 (Bossi and Dewey, 1992) and visualized in a secondary step using a μ-specific

goat anti-mouse antibody conjugated to FITC (Cat No 1020 - 02, Southern Biotechnology Associates, Birmingham, Al., USA). Mycelium of *U. atrum* in the sections was labelled specifically using the monoclonal antibody (MAb) Ua-PC3 (obtained from F.M. Dewey, Oxford University, Oxford, UK) and visualized in a secondary step using a γ-specific goat anti-rat antibody conjugated to TRITC (Cat No 3030 - 03, Southern Biotechnology Associates, Birmingham, Al., USA). Ua-PC3 is a rat IgG MAb raised against the surface washings of *U. atrum* mycelium. Ua-PC3 recognizes an antigen located in the cell wall of *U. atrum* and does not attach to *B. cinerea* mycelium (N. Karpovich and F.M. Dewey, unpubl.).
Mycelial colonization was quantified by measuring the relative surface area of the labelled mycelium in the section using image analysis. The measured relative surface area of the mycelium was mathematically converted into the relative volume of the mycelium in the section (Chapter 2).

Interacting colonies after point inoculation

Necrotic cyclamen leaves were competitively colonized by expanding colonies of *B. cinerea* and *U. atrum* after pairwise point inoculation with conidia. The outcome of this confrontation of mycelia was assessed after two weeks incubation by evaluating the sporulation formed on the leaf.
Sterile necrotic cyclamen leaves were re-hydrated overnight in sterile tap water at 4°C. Excess water was removed by blotting the leaves on sterile filter paper and the leaves were placed in petri dishes (55 mm in diameter, one leaf per dish, five petri dishes) on top of two sterile filter papers (50 mm in diameter) moistened with 0.6 ml of sterile tap water. Leaves were point inoculated on the adaxial side using a glass stamp, 0.5 mm in diameter. The sterile tip of the glass stamp was dipped in a conidial suspension of *B. cinerea* ($2x10^7$ conidia/ml) or *U. atrum* ($2x10^6$ conidia/ml) before each inoculation. Both species were inoculated at opposing ends of the leaf. On average 18 conidia of *B. cinerea* or *U. atrum* were deposited per inoculation site. After inoculation petri dishes were incubated at 18°C in the dark. After two weeks incubation the sporulation of both fungi inside and outside the macroscopically visible colonies was visually evaluated and reproduced in a drawing of each leaf using a dissecting microscope at 100X magnification.

Dynamic simulation model

In a theoretical approach, a dynamic simulation model was constructed in which internal mycelial biomass of *B. cinerea* and *U. atrum* grows logistically, limited by the availability of resources. Two types of resources were distinguished: 1) general resources always available to both species during intra- and interspecific competition and 2) optional species specific resources available to one of both species during intraspecific competition. The following equations were used to describe fungal biomass growth and resource utilisation:

$$\frac{dU}{dt} = r_U \cdot U \cdot (R + R_U) \qquad \text{(equation 3.1)}$$

$$\frac{dB}{dt} = r_B \cdot B \cdot (R + R_B) \qquad \text{(equation 3.2)}$$

$$\frac{dR}{dt} = -\mu \cdot \frac{dU}{dt} \cdot \frac{R}{R + R_U} - \beta \cdot \frac{dB}{dt} \cdot \frac{R}{R + R_B} \qquad \text{(equation 3.3)}$$

$$\frac{dR_U}{dt} = -\mu \cdot \frac{dU}{dt} \cdot \frac{R_U}{R + R_U} \qquad \text{(equation 3.4)}$$

$$\frac{dR_B}{dt} = -\beta \cdot \frac{dB}{dt} \cdot \frac{R_B}{R + R_B} \qquad \text{(equation 3.5)}$$

U = biomass of *U. atrum* relative to the single species carrying capacity [-], B = biomass of *B. cinerea* relative to the single species carrying capacity [-], R = amount of resources available to both, *U. atrum* and *B. cinerea*, scaled to 1 [-], R_U = relative amount of resources only available to *U. atrum* [-], R_B = relative amount of resources

only available to *B. cinerea* [-], r_U = relative growth rate of *U. atrum* [hour^{-1}], r_B = relative growth rate of *B. cinerea* [hour^{-1}], μ= resource use efficiency of *U. atrum* [-] and β= resource use efficiency of *B. cinerea* [-]. At t=0: $U=U_0$, $B=B_0$ and $R=R_0=1$. The model was implemented using the Fortran Simulation Translator, FST (Rappoldt and Van Kraalingen, 1996).

Growth of biomass of both fungi was simulated until all resources were depleted. Experimental results of the head start experiments were compared with hypothetical scenario's under the assumption that the final fungal biomass level reached in the simulations was proportional to sporulation. Equal interspecific and intraspecific competition was used as a reference scenario. This scenario was implemented using the following parameter settings: $R = 1$; $R_B = R_U = 0$; $r_U = r_B = 0.1$; $U_0 = B_0 = 0.01$ and $\mu = \beta = 1$. In a second step the effect of differential relative growth rates (r_u and/or r_b = 0.1, 0.2 or 0.3), differential resources (R_B and/or R_U = 0, 0.1 or 0.2) and differences in the initial biomass generated by germinating conidia (U_0 and/or B_0 = 0.0001, 0.001 or 0.01) was simulated. Finally, a scenario based on experimental estimates of the model parameters was implemented. Results of all simulated scenario's were compared with the reference scenario and experimental results of the head start experiment.

Statistics

The shape of the experimentally determined RY and RYT lines from the head start experiments was compared with simulated RY and RYT lines from the dynamic simulation model simulating growth of *B. cinerea* and *U. atrum* biomass based on nutrient competition as the dominant competitive mechanism. For this comparison it was assumed that the final fungal biomass level reached in the simulations was proportional to sporulation. Standard errors of the experimental average RY and RYT values were used to indicate significant differences between the simulated reference RY and RYT values and experimental RY and RYT values.

In the experiments on the dynamics of internal mycelial colonization and sporulation, logistic curves were fitted to the data describing sporulation development in time. For each experiment and fungal species a logistic curve was fitted. The lower asymptote was forced to zero. The relation between the internal mycelial colonization and time was analysed by fitting straight lines to log-transformed colonization data obtained in experiment 1 and 2. Only data from the first 60 hours of incubation were included in this analysis. Data from experiments 3

and 4 did not contain enough data points in the 0 - 60 hours interval to obtain reliable results. All analyses were performed with the statistical package Genstat 5 (Numerical Algorithms Group, Oxford, UK).

Results

Head start experiments

The yield (sporulation) of monocultures of *B. cinerea* and *U. atrum* on necrotic cyclamen leaf tissue was not influenced by differences in total incubation time caused by the different application intervals (dotted lines in Fig. 3.1A). This indicates that sporulation was fully developed and stable for all treatments after two weeks incubation counting from the first spray application. Standard errors of the average sporulation levels (SPLACI) indicate that the sporulation of *B. cinerea* was not significantly reduced by the presence of the antagonist when *B. cinerea* and *U. atrum* were applied simultaneously or when *B. cinerea* was given an application advantage on *U. atrum*. With increasing application advantages for *U. atrum*, *B. cinerea* sporulation was reduced until it was completely suppressed at advantages of 48 hours or more for *U. atrum* (Fig. 3.1A).
Sporulation of *U. atrum* was not significantly reduced by *B. cinerea* when *U. atrum* had an application advantage of 12 hours or more. With increasing application advantages for *B. cinerea*, sporulation of *U. atrum* decreased but *B. cinerea* was not capable of completely suppressing sporulation of *U. atrum* at the application intervals tested. *U. atrum* sporulation stabilized at a low level in the interval of 12 - 24 hours advantage for *B. cinerea*, independent of the dominating presence of *B. cinerea* (Fig. 3.1A).
The relative yields (RY) of *U. atrum* and *B. cinerea* increased with growing application advantages. The intersection between both RY lines was displaced upward and slightly to the right when compared to the reference position of the intersection for equal competitive abilities at 0 hours and 50% relative yield (Fig. 3.1B). As indicated by the standard errors of the average relative yield totals (RYT), the RYT was significantly higher than 1 for application intervals of 0 and 12 hours advantage for *U. atrum*. For longer or shorter advantages for *U. atrum* the RYT did not differ

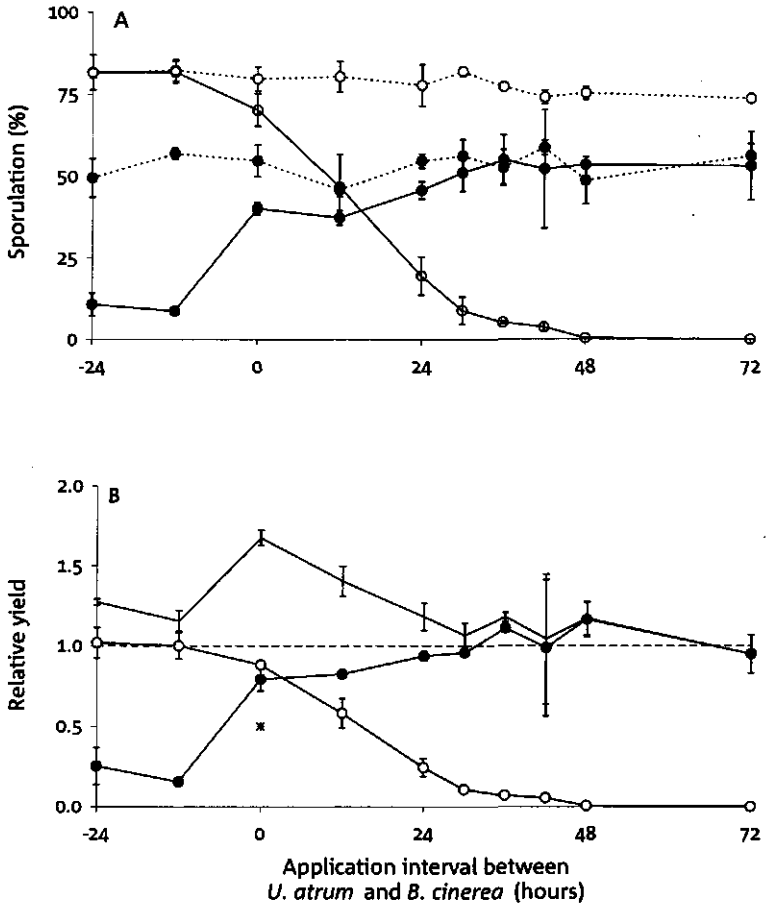

Figure 3.1. A: Sporulation measured as SPLACI (0 - 100%) of *Botrytis cinerea* and *Ulocladium atrum* on sterilized necrotic cyclamen leaf tissue after two weeks incubation at 18°C. Leaves were sprayed with conidial suspensions (1x10^6 conidia/ml) of *B. cinerea* and *U. atrum*, *B. cinerea* and sterile tap water containing 0.01% Tween 80 or *U. atrum* and sterile tap water containing 0.01% Tween 80 at different application intervals ranging from 24 hours application advantage for *B. cinerea* (-24 hours) to 72 hours application advantage for *U. atrum* (72 hours). (- -●- -) = *U. atrum* sporulation in monoculture. (- -○- -) = *B. cinerea* sporulation in monoculture. (—●—) = *U. atrum* sporulation in mixed culture with *B. cinerea*. (—○—) = *B. cinerea* sporulation in mixed culture with *U. atrum*.
B: Relative yields (RY, average sporulation in mixed culture divided by the average sporulation in the corresponding monoculture) and relative yield total (RYT) for sporulation of *U. atrum* and *B. cinerea* in mixed cultures. (—●—) = Experimental RY of *U. atrum*. (—○—) = Experimental RY of *B. cinerea*. (——) = Experimental RYT. (- - - -) = Theoretical RYT in case of equal intra- and interspecific competition. (*) = Theoretical intersection of RY lines for *B. cinerea* and *U. atrum* in case of equal

significantly from 1. The convex shape of the RYT line indicates stronger intraspecific competition than interspecific competition, i.e. some degree of niche differentiation between both fungi.

Dynamics of internal mycelial colonization and sporulation

Internal mycelial colonization

Large standard errors of mean colonization levels, found within and between experiments make it difficult to unequivocally interpret the results and extract the system characteristics. Overall, internal mycelial biomass followed a sigmoidal time course (Fig. 3.2).

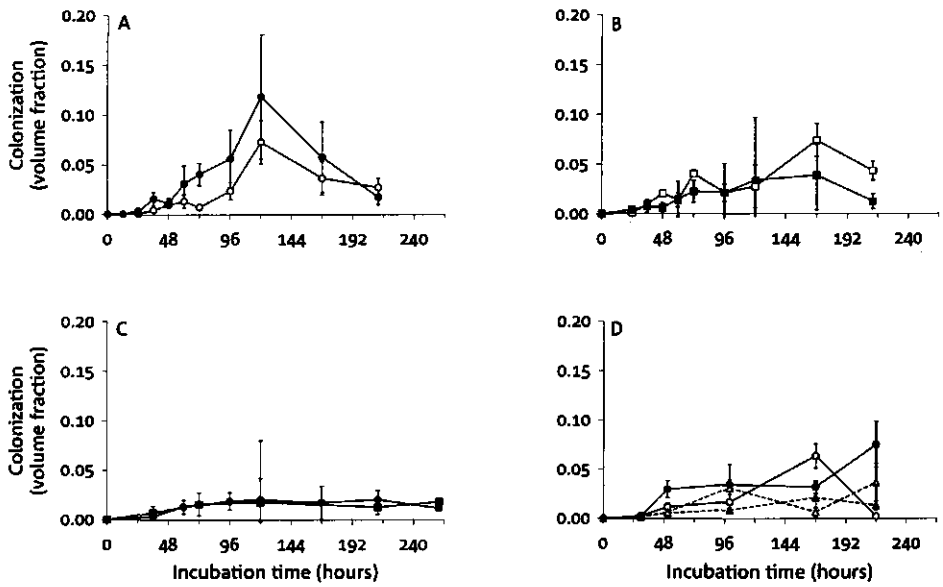

Figure 3.2. Internal mycelial colonization of sterilized necrotic cyclamen leaves in time by *U. atrum* and *B. cinerea* in colonization experiments 1 - 4 (A, B, C and D respectively). (—●—) =monoculture of *U. atrum* at 18°C. (—○—) = monoculture of *B. cinerea* at 18°C. (—■—) = monoculture of *U. atrum* at 24°C. (—□—) = monoculture of *B. cinerea* at 24°C. (- - -▲- - -) = *U. atrum* at 18°C in mixed culture with *B. cinerea*. (- - -Δ- - -) = *B. cinerea* at 18°C in mixed culture with *U. atrum*. Error bars represent the standard error of the mean.

However, three factors were complicating this analysis. 1) standard errors of the mean colonization level increased with increasing levels of colonization and incubation time, 2) maximum colonization levels reached by both fungi were not stable between experiments and 3) in some cases colonization levels were observed to decrease during the latest phases of colonization. Deviations from a sigmoidal colonization pattern might be related to differences in leaf quality between leaves within a batch as reported in Chapter 2. Differences between experiments might be related to differences in leaf quality between batches. Leaves for experiments 1 and 2 were picked in December, leaves used in experiments 3 and 4 were picked in April and February, respectively. The decrease of colonization levels during later phases of colonization was putatively caused by lysis of the hyphal elements.

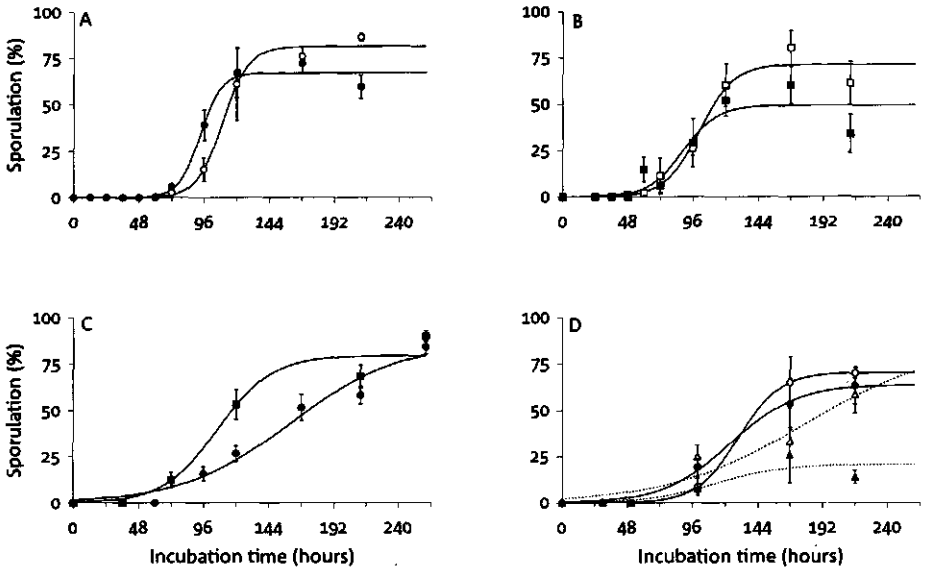

Figure 3.3. Sporulation measured as SPLACI (0 - 100%) of *U. atrum* and *B. cinerea* developing on sterilized necrotic cyclamen leaves in time in colonization experiments 1 - 4 (A, B, C and D respectively). (—●—) = monoculture of *U. atrum* at 18°C. (—○—) = monoculture of *B. cinerea* at 18°C. (—■—) = monoculture of *U. atrum* at 24°C. (—□—) = monoculture of *B. cinerea* at 24°C. (- - -▲- - -) = *U. atrum* at 18°C in mixed culture with *B. cinerea*. (- - -Δ- - -) = *B. cinerea* at 18°C in mixed culture with *U. atrum*. Error bars represent the standard error of the mean.

For *B. cinerea* and *U. atrum* it was observed that in the latest phases of colonization the clearly visible fluorescing hyphae were replaced by a halo of fluorescence without any apparent structure as if the hyphae had dissolved and the antigen had dispersed in the tissue (Fig. 3.4). Observations on even later phases of colonization were not possible due to high levels of maceration of the cyclamen tissue.
During the first 60 hours of colonization, colonization levels displayed a consistent exponential increase. No significant effects of fungal species or temperature on the colonization rate were detected (Table 3.2, Fig. 3.2B & 3.2C).
During competitive substrate colonization, fungal colonization dynamics followed the same patterns as in monoculture with a trend towards lower maximum colonization levels in mixed cultures than in monocultures (Fig. 3.2D).

Sporulation

In general, the sporulation on the leaves developed following a logistic pattern (Fig. 3.3). No systematic differences were observed in sporulation development of monocultures of *B. cinerea* and *U. atrum* (Table 3.3, Fig. 3.3A & 3.3B). Visually, sporulation was observed slightly earlier at 24°C then at 18°C for both fungi but the regression analysis revealed no significant differences between sporulation

Table 3.2. Estimated parameter values and corresponding standard error of the mean for straight lines fitted to log-transformed colonization data from the 0 - 60 hours incubation interval obtained in experiments 1 and 2 on the dynamics of internal mycelial colonization and sporulation of *Botrytis cinerea* and *Ulocladium atrum* in sterile necrotic cyclamen leaves.

		Ulocladium atrum				*Botrytis cinerea*			
		Slope[1]		Intercept[2]		Slope		Intercept	
Temperature	Experiment	Est.[3]	SEM[4]	Est.	SEM	Est.	SEM	Est.	SEM
18°C	1	0.056	0.021	-6.8	0.9	0.07	0.018	-8.2	0.8
24°C	2	0.031	0.013	-6.2	0.6	0.073	0.034	-8.0	1.5

[1]: Relative growth rate of fungal biomass (hour^{-1}).

[2]: Intercept of regression line of log-transformed colonization level against time with Y-axis.

[3]: Estimated value of the parameter.

[4]: Standard error of the parameter estimate.

Table 3.3. Estimated parameter values and corresponding standard error for logistic curves fitted to sporulation data obtained in experiments on the dynamics of internal mycelial colonization and sporulation of *Botrytis cinerea* and *Ulocladium atrum* on sterile necrotic cyclamen leaves.

		Ulocladium atrum						*Botrytis cinerea*					
		Slope[1]		Inflection point[2]		Upper asymptote[3]		Slope[1]		Inflection point[2]		Upper asymptote[3]	
Temperature	Experiment	Est[4]	SE[5]	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE
18 (°C)	1	0.127	0.03	93	1.8	67	2.1	0.105	0.01	110	1.4	81.4	1.9
	3	0.025	0	159.9	17.1	86.1	11.4	a	a	a	a	a	a
	4 monoculture	0.041	0	123.8	6.3	63.8	3.2	0.07	0	130	0.5	70.2	0.2
	mixed culture	0.041	b	112.7	b	20.6	b	0.02	0.01	179	90.1	85	65.8
24 (°C)	2	0.08	0.04	88.7	8.5	49.2	5.8	0.08	0.02	101	3.9	71.1	4.1
	3	0.05	0.02	106.2	9.5	79.5	6.4	a	a	a	a	a	a

[1] Sporulation development rate (hour^{-1}).

[2] Inflection point of the fitted logistic curve (hour).

[3] Maximum sporulation level or upper asymptote of the fitted logistic curve (SPLACI).

[4] Estimated value of the parameter.

[5] Standard error.

a Not included in the experiment.

b Standard error could not be estimated.

development at 18°C and at 24°C (Table 3.3). During competitive colonization, sporulation in mixed cultures developed in a similar fashion as in monoculture during the first 100 hours of colonization. Later on, sporulation of *U. atrum* in mixed cultures stayed behind the sporulation in monocultures. This effect was less pronounced or absent for *B. cinerea* (Table 3.3, Fig. 3.3D).

Internal mycelial colonization versus sporulation

The quantitative relationship between internal mycelial colonization and sporulation changed with incubation time. Early in the colonization process the internal mycelium did not yet support sporulation. When mycelial colonization levels decreased during the last phase of colonization, high levels of sporulation were supported by low levels of mycelial colonization. At 168 hours of incubation both sporulation and internal mycelial colonization had reached their maximum levels (Fig. 3.2 and Fig. 3.3) making this the most suitable point to study the relationship between sporulation and internal mycelial colonization.
From the scatter plots (Fig. 3.5) a weak trend can be detected that higher levels of mycelial colonization at 168 hours supported higher levels of sporulation. High levels of internal mycelial colonization always support high levels of sporulation but low levels of internal mycelial colonization can support high as well as low levels of sporulation. The orientation of the cloud of data points in both scatter plots is however markedly different. *B. cinerea* clearly displays the capability to safeguard its reproductive output even without a substantial mycelial presence. *U. atrum* also safeguards its reproductive output but low levels of internal mycelial colonization are more frequently linked to low levels of sporulation.

Interaction after point inoculation

Circular, radially growing colonies formed after point inoculation. After two weeks incubation the colonies of both species had colonized the entire leaf and were densely sporulating. A sharp demarcation line was visible between both colonies as if the confrontation between both mycelia had resulted in a deadlock after the mycelial contact. Detailed observation revealed however that *U. atrum* was also sporulating at low density within the entire *B. cinerea* colony. *B. cinerea* sporulation was not found in the *U. atrum* colony.

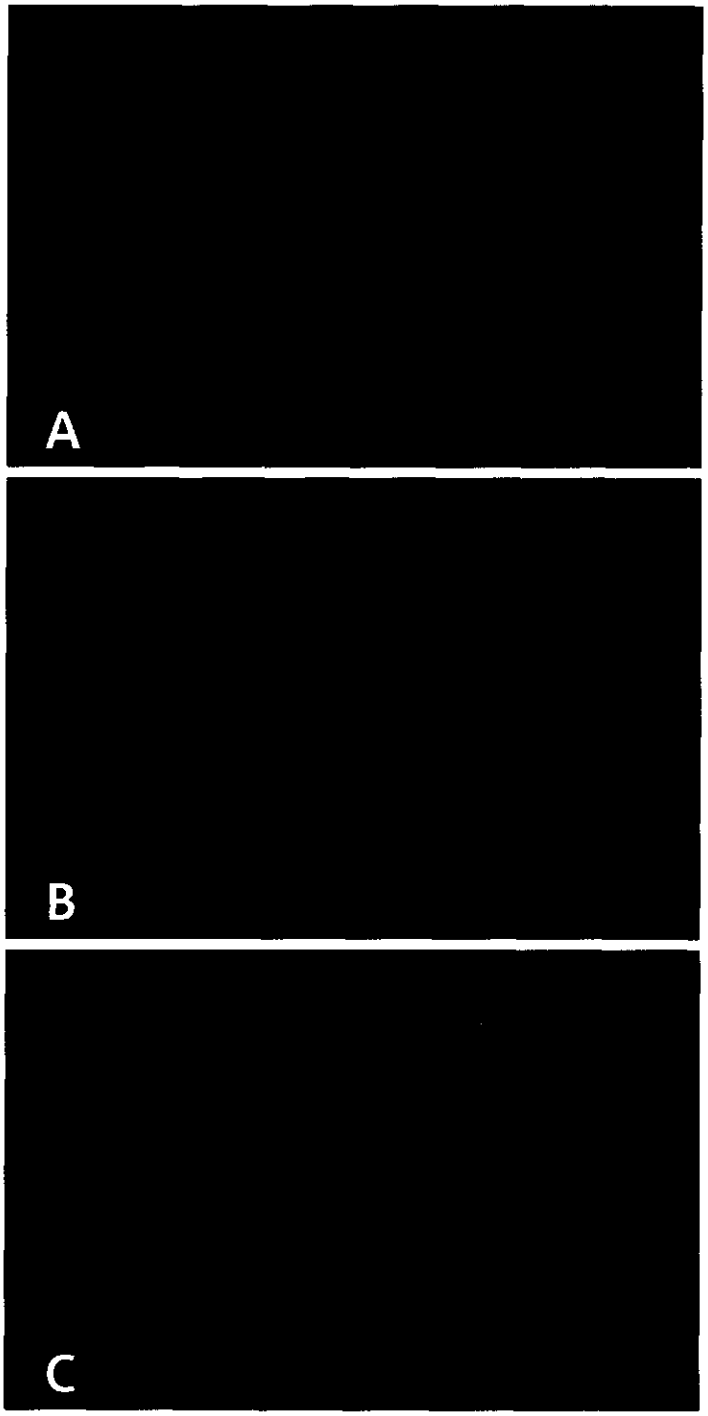

Figure 3.4. Photographs of cryostat sections of sterile necrotic cyclamen leaf tissue inoculated with conidia of *B. cinerea* after 51 (A), 168 (B) and 216 (C) hours of incubation at 18°C. Leaves were homogeneously inoculated with *B. cinerea* conidia at t=0 hours. *B. cinerea* hyphae were labelled with the *Botrytis* monoclonal antibody Bc-KH4 and visualized using a secondary antibody conjugated to the green fluorochrome FITC. *B. cinerea* colonization increases with time (A and B). During the latest phase of colonization (C) *B. cinerea* hyphae dissolve, leaving behind a halo of FITC fluorescence.

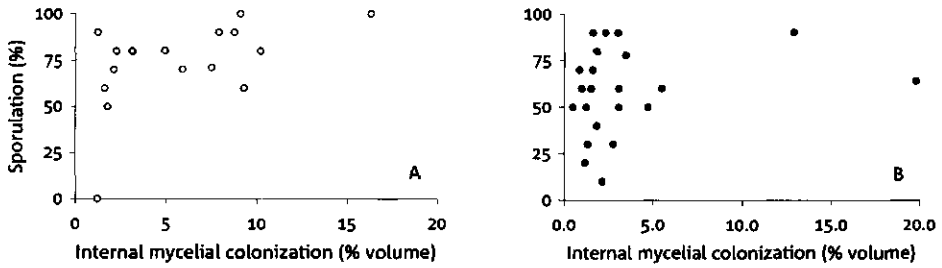

Figure 3.5. Relationship between internal mycelial colonization and sporulation, measured as SPLACI (0 - 100%), for monocultures of *B. cinerea* (A) or *U. atrum* (B) on necrotic cyclamen leaves. Internal mycelial colonization and sporulation were measured pairwise on the same leaves. Leaves were homogeneously inoculated with conidial suspensions and incubated for 168 hours at 18°C. Data were derived from experiments 1, 2, 3 and 4 (Table 3.1).

Simulation

Under the assumption that the final fungal biomass level reached in the simulations is proportional to sporulation, the general behaviour of the simulation model was in agreement with experimental results from the head start experiments. Simulated and measured RY lines displayed a sigmoid shape over the range of application intervals (Fig. 3.6A). The simulated reference scenario of equal inter and intraspecific competition (Fig. 3.6A) resulted in a close resemblance of the simulated and measured RY's for *U. atrum*. The simulated RY's for *B. cinerea* however were underestimated in this scenario. A major difference between the experimental results and the simulated results was found in the shape of the RYT line. The experimental RYT line was convex whereas the simulated RYT lines were straight horizontal lines. The effect of small changes of the model parameters, giving one of the fungi a competitive advantage, on the simulation results is summarized in Fig. 3.6. The response of the model system is described using five characteristics of the RY curves and RYT : 1) the steepness of both RY curves, 2) the position of the intersection of both RY curves, 3) the value of the RYT, 4) the lowest RY achieved and 5) the head start needed by each of the fungi to completely suppress the competitor. The relative growth rates (r_U and r_B) are the only parameters controlling the steepness of the RY curves. Higher relative growth rates resulted in shorter head starts needed for complete exclusion of the competitor (Fig. 3.6B). Differences in the relative growth rate of *U. atrum* and *B. cinerea* result in a lateral displacement of the intersection

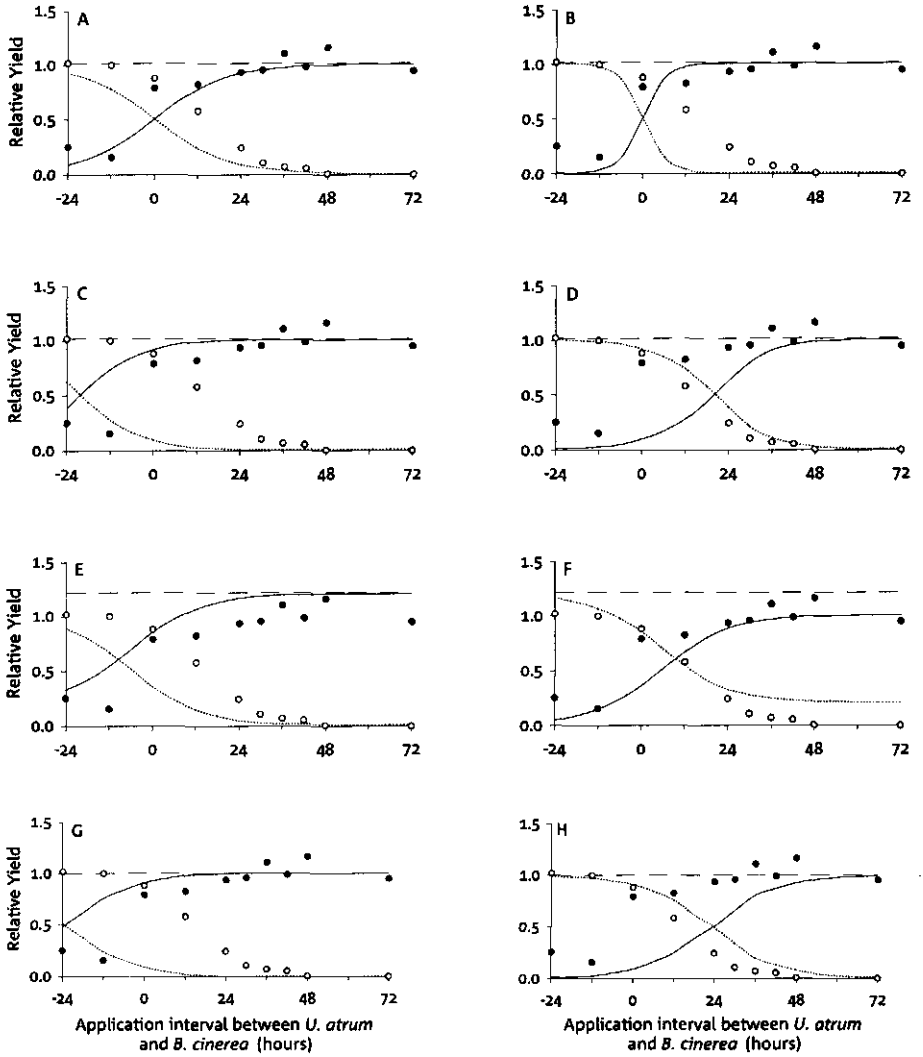

Figure 3.6. Relative yield (RY) of *B. cinerea* and *U. atrum* colonizing necrotic cyclamen leaves in simulated and experimental head start experiments. (●) = *U. atrum* experimental RY. (○) = *B. cinerea* experimental RY. (- - - -) = *B. cinerea* simulated RY. (——) = *U. atrum* simulated RY. (— —) = simulated RYT. **A:** Reference scenario with equal inter- and intraspecific competition. Model parameters: R = 1; $R_B = R_U = 0$, $r_U = r_B = 0.1$; $U_0 = B_0 = 0.01$ and $\mu = \beta = 1$. All other figures were generated with identical model parameters except for the following: **B:** $r_U = r_B = 0.3$. **C:** $r_U = 0.2$, $r_B = 0.1$. **D:** $r_U = 0.1$, $r_B = 0.2$. **E:** $R_B = 0$, $R_U = 0.2$. **F:** $R_B = 0.2$, $R_U = 0$. **G:** $U_0 = 0.001$, $B_0 = 0.0001$. **H:** $U_0 = 0.0001$, $B_0 = 0.001$. Experimental RY's and RYT are based on sporulation, simulated RY's and RYT are based on internal mycelial biomass.

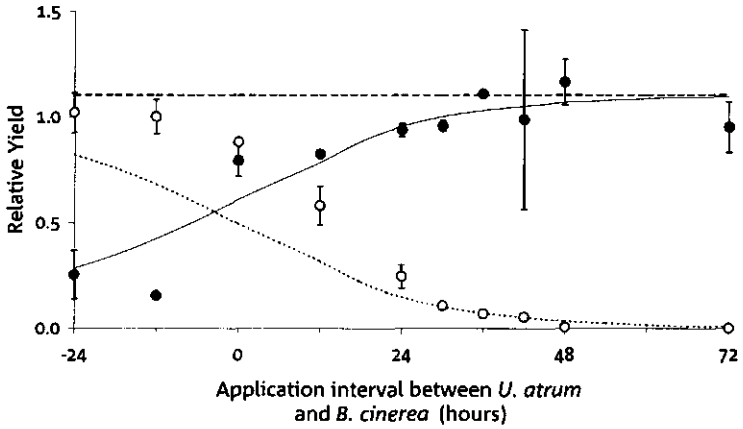

Figure 3.7. Relative yield (RY) of *B. cinerea* and *U. atrum* colonizing necrotic cyclamen leaves in a simulated and experimental head start experiments. The simulation was based on parameter estimates derived from experimental data on temporal mycelial colonization. (●) = *U. atrum* experimental RY. (○) = *B. cinerea* experimental RY. {- - - -} = *B. cinerea* simulated RY. (——) = *U. atrum* simulated RY. (— —) = simulated RYT. Model parameters were: R = 1; R_B = 0, R_U = 0.1, r_u = 0.056, r_b = 0.070, U_0 = 0.0020, B_0 = 0.0005. Experimental RY's and RYT are based on sporulation, simulated RY's and RYT are based on internal mycelial biomass.

between both RY curves (Fig. 3.6C & 3.6D). Changes in the relative growth rates did not influence the value of the RYT or the minimum colonization level achieved.
The availability of species specific resources ($R_B \neq 0$ and / or $R_U \neq 0$) raised both the minimum colonization level of the species to whom the specific resources were accessible and the RYT. Availability of species specific resources also caused the intersection between both RY curves to shift laterally and upward (Fig. 3.6E and 3.6F). Extra species specific resources did only marginally affect the steepness of both RY curves.
Differences in initial biomass for both fungi caused the intersection between both RY lines to shift laterally (Fig. 3.6G and 3.6H). The head start needed for complete suppression of the competitor shifted laterally along with the intersection of the RY lines. Minimum colonization levels and the RYT value were not influenced by differences in initial biomass.
Growth parameters for the last and most realistic scenario (Fig. 3.7) were based on the results of the regression analysis in Table 3.2: r_u = 0.056 [hour^{-1}], r_b = 0.070 [hour^{-1}], U_0 = 0.0020 [-], B_0 = 0.0005 [-]. Parameters determining the resource availability

were estimated from Fig. 3.1B: R = 1 [-], R_U = 0.1 [-], R_B = 0 [-]. The simulation did however not result in a close resemblance between experimental and simulated results (Fig. 3.7). Experimental RY curves are steeper and their intersection is displaced to the right and upward. In contrast, the intersection between the simulated RY curves is displaced to the left and upward but it does not match the experimental intersection. The simulated RYT curve is a straight horizontal line lacking the typical convex shape of the experimental RYT line.

Discussion

Our goal was to elucidate mechanistic principles behind the competitive interaction between *B. cinerea* and *U. atrum* in necrotic cyclamen leaves and to gain quantitative information on competitive and non-competitive substrate colonization by both fungi. Processes at leaf level are of particular importance for biocontrol because of the abundance of mycelial colonization and sporulation found on leaves which determine the pathogen's potential for dispersal within and between plants. Processes at tissue level underlie the patterns observed at leaf level and are thus important to understand the dynamics of fungal colonization and sporulation.

The dynamics of internal mycelial colonization were found to follow logistic growth patterns. The logistic equation is the most widely used equation to describe the evolution of biomass in fungal solid state fermentation (Smits, 1998). However, large standard errors and an observed decline of colonization levels in the latest phases of the process urge for some reservation regarding this conclusion. Logistic growth patterns of fungal biomass were previously reported for e.g. *Cladosporium fulvum* in tomato leaves (Karpovich-Tate et al., 1998), for *Humicola lanuginosa* on rice grains (Dewey et al., 1992) and for *Rhizopus oligosporus* colonies on artificial medium (Nout et al., 1997). The large standard errors may be due to a variable nutritional status of leaves. Production of mycelial biomass, ageing of the fungus and sporulation are all influenced by the amount and quality of the nutrients available (Engelkes et al., 1997). Especially the carbon content and the carbon to nitrogen ratio were reported to influence fungal growth and development (Lockwood, 1981). The nutritional status of necrotic cyclamen leaves is likely to differ within (Chapter 2) and between batches of leaves. Leaf batches used in the experiments were harvested during the

period December - May in which day length is increasing. The principal nutrient sources in plant tissue after natural senescence include remaining cell wall components such as cellulose, hemicellulose and lignin. The level of amino acids and soluble sugars is low due to relocation of these substances during senescence (Baddeley, 1971). It cannot be ruled out that after artificial senescence, following leaf detachment, significant amounts of soluble sugars and amino acids stay behind and are available for fungal consumption. Nutritional factors thus constitute a significant source of variation. Experiments on defined artificial media could resolve this issue. *B. cinerea* and *U. atrum* display similar colonization patterns and maximum colonization levels within experiments. The nutritional status of the necrotic cyclamen leaves is thus equally stimulating or hampering both fungi (Fig. 3.2) and does not result in a competitive advantage for *B. cinerea* or *U. atrum*. Necrotic leaves in cyclamen- and especially in other crops are likely to differ significantly in their nutritional composition. Consequently this must be taken into account when assessing the perspectives for biocontrol of *B. cinerea* using *U. atrum*.

The decline in colonization levels observed after reaching carrying capacity was supported by visual observations (Fig. 3.4). Autolysis of cell walls, initiated in the ageing mycelium by nutrient deprivation as described for *Schizophyllum commune* (Wessels & Sietsema, 1984) is a likely cause of this phenomenon. Similar findings were reported for *Sclerotium rolfsii* (Edelstein et al., 1983) and *Rhizopus oligosporus* (Nout et al., 1997) but not for *Cladosporium fulvum* in tomato leaves (Karpovich-Tate et al., 1998), or for *Humicola lanuginosa* on rice grains (Dewey et al., 1992). Apart from the inherent characteristics of the fungal species, the technique used to quantify fungal biomass might cause these different findings. A decline of fungal biomass was always found using optical or harvesting techniques. In both cases where the decline was not found (Dewey et al., 1992; Karpovich-Tate et al., 1998) a quantitative ELISA was employed. The ELISA technique will still detect persistent fungal antigens dispersed in the substrate (Fig. 3.4c) after the hyphae themselves have been dissolved in a lytic process.

A clear quantitative relationship between sporulation and internal mycelial colonization could not be established (Fig. 3.5). High levels of mycelial colonization were not essential to produce high levels of sporulation. However, several factors complicate this quantitative analysis: 1) Visual quantification of sporulation, as was done here, was shown to significantly underestimate the number of conidia produced by *B. allii* at high sporulation intensities (Köhl et al., 1995c). 2) The quality

of the conidia as reflected in their size and internal nutrient reserves was not taken into account. 3) The internal mycelial biomass does not develop in a purely cumulative fashion. 4) The nutritional composition of the leaf and its effect on development of mycelial biomass and sporulation is unknown.

The mechanistic principles behind the interaction of *U. atrum* and *B. cinerea* were analysed using head start experiments and pairwise confrontations between the mycelia. Factors potentially influencing the outcome of a replacement experiment such as active host resistance, host susceptibility or pathogenicity factors (Newton et al., 1998) are unlikely to interfere in necrotic tissue. Potential effects of inoculum density on the competitive interaction (Firbank and Watkinson, 1985; Snaydon, 1991) were not taken into account. The chosen inoculum densities represented a worst case scenario for the antagonist with realistic conidial densities for *U. atrum* and unrealistically high conidial densities for *B. cinerea*.

Pairwise confrontations of mycelia of *U. atrum* and *B. cinerea* in necrotic cyclamen leaf tissue demonstrated that *U. atrum* was capable of invading the oldest parts of an established *B. cinerea* colony whereas the reverse did not occur. The relevance of this phenomenon for biocontrol purposes is limited since the pathogen's sporulation was not noticeably reduced. The production of consecutive crops of conidia by the pathogen might however be affected. With respect to resource utilization the results of the mycelial confrontation imply that the available resources for *U. atrum* in a necrotic cyclamen leaf include all the essential resources available to *B. cinerea* plus a small amount of resources specifically available to *U. atrum*. This conclusion is supported by the position of the two experimental RY lines and the RYT line in Fig. 3.1 which is located well above the reference line for equal inter- and intraspecific competition. Alternatively, *U. atrum* could benefit from by-products of enzymatic degradation of the tissue by *B. cinerea* which would otherwise not have been available to the antagonist.
The hypothesis on nutrient competition as the dominant antagonistic mechanism between *B. cinerea* and *U. atrum* and on the nutrient utilization spectra of both fungi were checked using simulation. In general, comparison of experimental and model results supported the hypothesis of nutrient competition as the dominant competitive mechanism between *U. atrum* and *B. cinerea*. A major difference between the model results and experimental results was found in the shape of the RYT line. The experimental RYT line (Fig. 3.1), based on sporulation, was convex whereas the model RYT lines (Fig. 3.6, 3.7), based on mycelial biomass, were

horizontal straight lines. The convex shape of the experimental RYT line indicates a less than proportional reduction of sporulation of *U. atrum* and *B. cinerea* in competitive situations (Fig. 3.1) and thus a stimulating effect of competition on sporulation. Alternatively, the convex shape of the RYT line could be caused by 1) by-products of enzymatic degradation of the tissue produced by one species and beneficial to the other (Rayner and Webber, 1984) or 2) differences in metabolic capabilities of the fungi during their development. The discrepancy between experimental and simulated results with realistic, experimentally determined parameters (Table 3.2, Fig. 3.7) further accentuates the unknown effects of competition on conidial reproduction and the lack of a clear quantitative relationship between (simulated) mycelial colonization and (experimentally determined) sporulation. The shape of the RYT line also indicates that toxins are unlikely to be involved in the competitive relationship between *U. atrum* and *B. cinerea*. In case toxins would have been involved the RYT line would be concave and fall below 1 (Fig. 3.1)(Braakhekke, 1980).

Cooke and Rayner (1984) and Rayner and Webber (1984) distinguish competitive, neutralistic and mutualistic mycelial interactions. Competitive interactions comprise interactions based on primary resource capture and combative interactions (Chapter 1). For the present system we find that: 1) B. *cinerea* and *U. atrum* both belong to the primary colonizers of necrotic plant tissue (Hudson, 1971; Köhl et al., 1995c). 2) Mycelial contact between both species does not negatively affect the mycelia (Köhl et al., 1997). 3) *U. atrum* and *B. cinerea* grow and sporulate in mixed cultures (Fig. 3.2, 3.3). 4) *U. atrum* pre-emptively colonizes necrotic cyclamen leaf tissue and excludes *B. cinerea*. 5) The outcome of the race for resources between *B. cinerea* and *U. atrum* in necrotic cyclamen tissue can be decided within the very first phase of the colonization process. All these facts point towards a competitive interaction based on primary resource capture. *U. atrum* captures some of the secondary resources left behind by *B. cinerea* but while doing so does not noticeably affect the primary reproductive output of the pathogen rendering this effect irrelevant for biocontrol purposes.

The outcome of interactions based on primary resource capture are determined by factors such as effective propagule dispersal, spore germination, mycelial extension, possession of suitable enzymes and tolerance to the adverse conditions associated with the resource (Rayner and Webber, 1984). This type of interaction in a biocontrol setting has the advantage that the biocontrol effect can be established well before the pathogen's arrival. On the other hand, curative effects of *U. atrum* against

B. cinerea are not to be expected. Timing of antagonist application therefore seems to be of critical importance to the desired biocontrol effect. The ability of *U. atrum* conidia to survive for extensive periods in the phyllosphere (Elmer and Köhl, 1998; Köhl et al., 1998) and its ability to rapidly colonize a substrate in a climatological window of opportunity might thus be two of the most valuable characteristics that make *U. atrum* an effective biocontrol agent.

Chapter 4

The role of substrate specificity in biological control of *Botrytis elliptica* in lily and *Botrytis cinerea* in cyclamen with *Ulocladium atrum;* a comparative study

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Submitted.

Abstract

The fungal antagonist *Ulocladium atrum* is as effective against *Botrytis cinerea* in cyclamen under commercial growing conditions as standard fungicide treatments. *U. atrum* is not very effective against *Botrytis elliptica* in lily. In a comparative study at laboratory and field level, this difference in effectiveness is explained in terms of substrate specificity of antagonist and pathogen and in terms of infection pathways of the pathogens towards infection of the healthy host plant. The saprophytic *U. atrum* is capable of excluding necrotrophic *Botrytis* spp. from necrotic tissue, their mutual substrate. In cyclamen, *B. cinerea* initially is only weakly pathogenic and has to go through a saprophytic phase in necrotic tissue before its mycelium can infect healthy plant parts. *U. atrum* is effective against *B. cinerea* in cyclamen because it excludes *B. cinerea* from necrotic tissue.
B. elliptica is more pathogenic than *B. cinerea* with conidia and mycelium capable of directly infecting healthy lily leaf tissue. *U. atrum* applications aimed at blocking the infection pathway utilizing necrotic tissue are therefore not effective against *B. elliptica*. Control options based on competitive interactions in and around *B. elliptica* lesions also proved ineffective.
The results demonstrate that the potential of *U. atrum* as a biocontrol agent against *Botrytis* spp. and possibly against other necrotrophes is determined by the competitive ability of the antagonist in mutual substrates of pathogen and antagonist and by the role of these mutual substrates in the epidemiology of the pathogen.

Introduction

Botrytis spp. cause economically important diseases in numerous greenhouse and field crops. *Botrytis elliptica* (Berk.) Cooke, the causal agent of "lily fire" has been responsible for economically significant losses in lily production (Doss et al., 1984). *Botrytis cinerea* Pers. ex Pers. is a major pathogen in many vegetable-, flower- and fruit crops. It causes leaf rot in cyclamen and is considered to be one of the major pathogens in this crop.
At present, the control of *Botrytis* diseases is based on the frequent use of fungicides. However, *Botrytis* spp. have shown a great potential to develop fungicide resistance (Gullino, 1992; Migheli et al., 1990). In addition, an increasing concern about the effects of pesticide residues on environment and human health (Jansma et al., 1993) leads to an increasing number of restrictions on the use of pesticides. Biological control offers an environmentally friendly supplement or alternative to chemical control.

Necrotrophic pathogens such as *Botrytis* spp. colonize plant tissue killed either by the pathogen itself or by other factors. Sporulation occurs exclusively on necrotic tissue. The host plant range of *B. elliptica* is practically limited to lily (*Lilium* spp.) although alternative hosts have been reported (MacLean, 1948). In contrast, *B. cinerea* has a host plant range of over 200 species (Jarvis, 1977; 1980a).

B. elliptica is spreading within and between lily plants through contact infections and splash or wind dispersal of conidia (Beyma thoe Kingma and van Hell, 1931). *B. elliptica* infection of lily leaves results in brown expanding lesions. Associated with the lesion, yellow streaks of senescing tissue can be formed, distally and proximally from lesion. Formation of yellow streaks is not specific for the lily - *B. elliptica* interaction and can be evoked by inoculation with several other micro-organisms and by detaching leaves from the plant (Doss et al., 1988). Yellow streaks grow longer and wider with the expanding lesion. Eventually the lesion will cover the full width of the leaf, block all vascular transport and kill the tissue distally from the lesion well ahead of the advancing *B. elliptica* mycelial front. From the leaves *B. elliptica* migrates to the stem, eventually blocking vascular transport and killing plant parts located above the location of stem infection (Beyma thoe Kingma and van Hell, 1931).

Similar to the situation in many other crops (Jarvis, 1980b), *B. cinerea* is spreading within and between cyclamen plants through mycelial infections and splash or wind dispersal of conidia. *B. cinerea* infection of cyclamen results in expanding lesions on petiole or leaf blade. Infection of the petiole blocks all vascular transport to and from the leaf blade killing the tissue distally of the site of the lesion, well ahead of the mycelial colonization front.

The saprophytic fungal antagonist *Ulocladium atrum* Preuss, isolate 385 is capable of suppressing sporulation of *Botrytis* spp. in several necrotic tissues (Köhl et al., 1995b ; 1995c) resulting in suppression of *Botrytis* spp. sporulation. Reduction of sporulation of *Botrytis* causes a delay in the disease build up as demonstrated in onion (Köhl et al., 1995a). However, very different results have been achieved in biocontrol experiments with *U. atrum* applied against *B. elliptica* or applied against *B. cinerea*. *U. atrum* is successfully suppressing *Botrytis* spp. in laboratory assays on sterilized necrotic leaf tissue (Köhl et al., 1995c) and on sterilized necrotic leaf tissue exposed to field conditions (Köhl et al., 1995b). In cyclamen, *U. atrum* is even as effective as standard fungicide applications against *B. cinerea* in commercially grown crops (Köhl et al., 1998). *U. atrum* applications against *B. elliptica* in field grown lily crops have however not resulted in a pronounced effect on *B. elliptica* epidemics (Köhl, unpubl.).

Biological control of *Botrytis* spp. by *U. atrum* aims at the interaction between *Botrytis* spp. and *U. atrum* within the plant tissue (substrate). Due to their respective necrotrophic and saprotrophic characteristics, the fungi have different ranges of potential substrates they can infect or colonize in each crop: conidia and mycelium of *B. elliptica* autonomously infect healthy lily tissue (Beyma thoe Kingma and van Hell, 1931; Doss et al., 1984). Conidia and mycelium of *B. cinerea* are often incapable of overcoming host plant defences without the presence of exogenous nutrients at the infection site (van den Heuvel, 1981; Schlösser 1978). *U. atrum* is a saprophytic fungus, typically found on decaying organic material and is not considered to be pathogenic to lily or cyclamen. The desired interaction between *Botrytis* spp. and *U. atrum* can only take place within a mutual substrate. For biological control of *Botrytis* spp. by *U. atrum* additional prerequisites have to be fulfilled. First, *U. atrum* has to be able to out compete *Botrytis* spp. in their mutual substrate. Second, this mutual substrate has to play a significant role in a developing epidemic caused by the *Botrytis* spp., either as a source of inoculum or as part of an infection pathway. In this paper we compare the pathosystems *B. cinerea* in cyclamen and *B. elliptica* in lily seeking to explain the differential biocontrol capabilities of the antagonist. In a laboratory experiment we first identify mutual substrates for pathogen and antagonist in lily and cyclamen. Second, we quantitatively monitor the availability of the identified mutual substrates for pathogen and antagonist in field grown lily- and greenhouse cyclamen crops. Third, we determine the potential effect of excluding the pathogen from the mutual substrate, simulating a perfectly effective antagonist, on epidemics caused by *Botrytis* spp. and compare this effect to the actual control effect of antagonist applications in field grown lilies and greenhouse cyclamen crops.

Materials and Methods

Fungal cultures

B. cinerea isolate 700, originally isolated from a diseased gerbera was cultured on oatmeal agar (20 g of milled oat, 15 g of agar and 1 L of tap water) for two weeks at 20°C in the dark. *B. elliptica*, isolate BE9401 originally isolated from a diseased lily, was cultured on malt extract agar (Oxoid CM59) for 2 weeks at 18°C under

continuous light of an 18W blacklight and an 18W cool-white fluorescent tube. *U. atrum* isolate 385, originally isolated from a necrotic leaf tip of a field grown onion was cultured on oatmeal agar for 4 weeks at 20°C in the dark. Conidial suspensions of all fungi were prepared by flooding the cultures with sterile tap water containing 0.01% Tween 80, detaching the conidia by gently rubbing with a rubber spatula and filtering of the suspension through a nylon gauze with a mesh of 200 µm. For greenhouse or field experiments, larger quantities of *U. atrum* 385 conidia were produced on oat grains in autoclavable spawn bags (type 3LS, van Leer Ltd., Poole, Dorset, UK) incubated for 4 weeks at 20°C in the dark (Köhl et al., 1998). Conidial suspensions were obtained by agitating the oat kernels in chilled tap water containing 0.01% Tween 80 in a small washing machine (Nova MW 100; Nova, Maastricht, the Netherlands) and filtering the suspension through a gauze with a 200 µm mesh. Conidial concentrations of all suspensions were determined using a haemocytometer and adjusted to the desired concentration.

Substrate specificity

Plant material

Substrate specificity of *B. elliptica*, *B. cinerea* and *U. atrum* was studied on five different tissue types of cyclamen (*B. cinerea*) and lily (*B. elliptica*). Asiatic hybrid lilies (*Lilium*) cv. Mont Blanc were grown in a greenhouse set at 18°C. This cultivar is generally considered to be very susceptible to *B. elliptica*. Cyclamen plants (*Cyclamen persicum* L.) cv. Superserie were grown in a greenhouse set at 20°C. In both crops, no additional lighting was provided and flower buds were removed to prevent pollen deposition on the leaves, which is known to enhance infection by *B. cinerea* (Chou and Preece 1968). No infections by *Botrytis* spp. or other diseases occurred on the leaves.

Treatments and experimental set up

Based on the necrotrophic and saprotrophic characteristics of the fungi, five substrates or tissue types were distinguished for lily and cyclamen: (1) healthy leaf tissue (green), (2) senescing leaf tissue (yellow but turgescent), (3) necrotic leaf tissue (yellow - brown without turgor) and necrotic leaf tissue pre-colonized by (4) *U. atrum*

and (5) *B. cinerea* (cyclamen leaves) or *B. elliptica* (lily leaves). Pre-colonized necrotic tissue was included as a model for *Botrytis* lesions or for tissue already colonized by the antagonist.

To produce pre-colonized tissue, healthy leaves were picked from greenhouse lily and cyclamen plants and dried slowly at room temperature to simulate natural senescence. After drying, leaves were sealed in small plastic bags, sterilized by gamma irradiation (4Mrad) and stored. Forty-eight hours prior to the start of the experiment the sterilized necrotic leaves were rehydrated, homogeneously spray inoculated with conidial suspensions ($1x10^6$ conidia/ml) of the respective fungi, placed on top of a sterile plastic grid in a sterile petri dish (90 mm in diameter) with two sterile filter papers (80 mm in diameter) moistened 1.5 ml of sterile water and incubated at 18°C in the dark.

At the start of the experiment, healthy, senescing and necrotic leaves were sampled from greenhouse lily and cyclamen plants and placed in petri dishes as described above. At this point, the petri dishes containing the pre-colonized tissue were included in the experiment. Each of the five tissue types of lily or cyclamen were homogeneously spray inoculated with conidial suspensions ($1x10^6$ conidia/ml) of *B. elliptica* or *B. cinerea* respectively, *U. atrum* or sterile tap water containing 0.01% Tween 80 using an atomizer (Desaga, Heidelberg, Germany) and incubated at 18°C in the dark for 48 hours. Inoculation resulted in an average conidial density of 85 conidia/mm^2 as determined by direct microscopical observations. Three leaves in one petri dish were used for each host - tissue type - inoculant combination.

Sampling, sectioning and immunolabelling.

Leaves were destructively sampled after 48 hours incubation. One sample, approximately 3x5 mm in size, per leaf was processed and sectioned using a cryostat (Chapter 2). Mycelium of *Botrytis* spp. in the sections was labelled specifically using the monoclonal antibody Bc-KH4 (Bossi and Dewey, 1992) conjugated to the green fluorochrome FITC. Mycelium of *U. atrum* in the sections was labelled specifically using the monoclonal antibody Ua-PC3 (Karpovich - Tate and Dewey, Oxford University, Oxford) conjugated to the red fluorochrome TRITC.

Observations

Just before sampling, after 48 hours incubation, the presence of lesions, external mycelium and sporulation was recorded using a dissecting microscope. Presence or absence of internal mycelium of *Botrytis* spp. or *U. atrum* was examined in the immunofluorescently labelled cryostat sections using fluorescence microscopy. Six - eight sections were examined per leaf. Abundance of internal mycelium was recorded as absent (–), a trace of mycelium present (+/-), low levels of colonization present throughout the sections (+) and abundant mycelium present throughout the sections (++).

Cyclamen greenhouse experiments

Three greenhouse experiments were carried out. Experiment 1 was used to monitor the availability of different tissue types in a commercial cyclamen crop. Experiments 2 and 3 were used to study the effect of *U. atrum* applications on *B. cinerea* epidemics and to test the hypothesis that saprophytic colonization of necrotic tissue is an essential step for *B. cinerea* towards infection of the healthy plant.

Plant material and growing conditions

In experiment 1, cyclamen plants (*Cyclamen persicum* L.) cv. Superserie, 20 weeks old, were placed on tables with capillary matting in a commercial greenhouse at a spacing just wide enough to avoid touching of the leaves.
For experiment 2 and 3, cyclamen plants, cv. Superserie, were obtained from a commercial grower at the age of 22 (experiment 2) or 10 weeks (experiment 3). The plants were not previously treated with pesticides. *B. cinerea* severity, assessed immediately after arrival of the plants, revealed an average of 0.1 leaves per plant sporulating with *B. cinerea* in experiment 2 and and 0 leaves per plant sporulating with *B. cinerea* in experiment 3. Plants were placed on tables with capillary matting at a spacing just wide enough to avoid contact between leaves. Greenhouse temperature varied within the 15 - 25°C range set as minimum and maximum temperature. Relative humidity of the air mostly varied between 80 - 90%.

Treatments

No treatments were applied to the plants of experiment 1.
Treatments in experiment 2 and 3 were: (1) spraying the plants with tap water containing 0.01% (vol/vol) Tween 80 every four weeks, (2) spraying with a conidial suspension of *U. atrum* ($1x10^6$ conidia/ml) every four weeks, (3) an untreated control and (4) a treatment in which senescing, visually non *B. cinerea* infected, plant parts were removed at 3 - 4 day intervals. In the latter treatment, necrotic plant parts and plant parts sporulating with *B. cinerea* were left on the plant. Experiment 2 included a fifth treatment: a single spray with a *U. atrum* conidial suspension($1x10^6$ conidia/ml), coinciding with the first spray application to other sprayed treatments. Spray treatments were applied at plant age 155, 183 and 211 days in experiment 2 and at 140, 168, 196 and 224 days in experiment 3. All plants were sprayed individually on- and inside the canopy to reach shielded surfaces such as older leaves, petioles and tuber. This technique is generally used to apply fungicides in commercial cyclamen crops.
In experiment 2, *B. cinerea* was not artificially introduced since it was naturally present from the start of the experiment. In experiment 3, *B. cinerea* was introduced by placing six sporulating cultures (3-4 weeks old), evenly spaced, in each of the greenhouse compartments (± 80 m^3) when the plants were 166 days old. The lids were removed for 48 hours during which time *B. cinerea* conidia were allowed to spread through the greenhouse compartments with the circulating air.

Experimental design

Experiment 1 was arranged in 4 replicate plots, each with 24 plants in four rows of six plants. All plants were located on the same table in a commercial greenhouse.
Experiments 2 and 3 were designed as randomized block experiments with 5 and 4 treatments respectively, each treatment being replicated 4 times. Two greenhouse compartments were used, each containing two blocks. Each replicate (plot) contained 25 plants arranged in 5 rows of 5 plants. Plots were separated 50 - 60 cm.

Observations

The availability of senescing and necrotic tissue was monitored in all plants of experiment 1 and in all plants of the untreated control plots in experiment 2. Eight different categories were distinguished for both petioles and leaf blades: senescing or necrotic, attached to the plant or detached from the plant and with or without sporulation of *B. cinerea*. Plant development was followed by counting the number of green leaves of three, randomly assigned, but fixed, plants per plot.
In experiments 2 and 3, the number of leaves per plant with sporulation of *B. cinerea* on petiole or leaf blade was assessed at two week intervals. The last assessment in experiment 2 took place three weeks after the previous assessment.

Crop protection

No pesticides were used. Cyclamen plants in experiment 2 were attacked by caterpillars of *Clepsis spectrana* (TR.) and by *Fusarium oxysporum* Schlecht.: Fr. f.sp. *cyclaminis* Gerlach. Caterpillars were removed by hand, plants severely damaged by caterpillars were also removed. Cyclamen plants infected by *Fusarium* were always removed from the plots. At the end of experiment 2, 37 plants (on a total of 500 plants) had been removed due to one of both causes. Aphids were controlled by two introductions of ladybirds (*Hippodamia convergens*; Hippopack, Brinkman BV, 's-Gravenzande, the Netherlands) in experiment 2 and 3.

Lily field experiments

Similar to the objectives of the greenhouse cyclamen experiments the objectives for both lily field experiments were to monitor the availability of necrotic tissue as the mutual substrate for *U. atrum* and *B. elliptica* in lily crops, to test whether naturally necrotic tissue constitutes an important infection pathway of *B. elliptica* in lily and to study the effect of *U. atrum* applications on *B. elliptica*.

Field experiment 1

Bulbs of Asiatic hybrid lilies (Lilium) cv. Mont Blanc were planted in April 1998. Eight plots of 150 plants were established in rows according to standard practice. Two treatments were included in a completely randomized design: (1) removing naturally necrotic leaves at 7 day intervals and (2) an untreated control. Treatments and observations were started on 27 May 1998 and stopped on 1 July 1998 when 80% of the leaves was infected by *B. elliptica*.
B. elliptica severity was assessed as the percentage of infected leaves of 10 randomly chosen but fixed plants per plot at weekly intervals. The number of naturally necrotic leaves was monitored in all plants per plot. From the removed leaves, a sample of 50 randomly chosen leaves was incubated in moist chambers at 20 °C under the combined continuous light of 3 fluorescent tubes (1x TLD 18W/08 and 2x TLD 18W/840) for 7 days to stimulate sporulation of the fungi present. After incubation sporulating *Botrytis* spp. were identified based on shape and size of the conidia (Ellis, 1971).

Field experiment 2

Plant material. Bulbs of Asiatic hybrid lilies (*Lilium*) cv. Mont Blanc were planted in April 1996. Twenty eight mini plots, with 4 rows of 5 plants per plot and 5 m distance between plots, were established within a sugar beet crop to minimize interplot interference.

Treatments. Plots were sprayed with a (1) *U. atrum* conidial suspension ($2x10^6$ conidia/ml) or (2) with water containing 0.01% Tween 80 at weekly intervals. Two starting dates for the spray treatments were included to study the effect of introducing the antagonist in different phases of an epidemic caused by *B. elliptica*. Treatments were started on 5 August 1996 and 19 August 1996. The last treatment was applied on 9 September 1996. Numerous primary infections of *B. elliptica* were found for the first time on 16 August 1996.

Experimental design. Experiment 2 was designed as a split spit plot design with two starting dates for the treatments, two spray treatments and two sampling positions within each plot. Starting dates were assigned to two main plots. Main plot 1 contained 6 plots, main plot 2 contained 22 plots. The two spray treatments were

assigned randomly to the plots within main plot 1 and 2 in 3 and 11 replicates respectively. Ten necrotic leaves were collected on 29 October 1996 from each plot and from each of two positions in the canopy: from the top of the canopy and from the lowest leaf layers of the canopy.

Observations. Sampled necrotic leaves were incubated for 5 days at 20°C in moist chambers to allow fungi present to sporulate. Fungal sporulation per leaf was quantified using a dissecting microscope distinguishing between *B. elliptica*, *Ulocladium* spp. and naturally occurring saprophytic *Cladosporium* spp. and *Alternaria* spp. The fraction of leaf covered with conidiophores and the relative intensity of the sporulation for each of the genera distinguished was assessed. Multiplication of the two parameters yields the Sporulating Leaf Area Corrected for Intensity (SPLACI, Köhl et al., 1998), the relative leaf area sporulating at maximum intensity.
To obtain an impression of the leaf area available for *U. atrum* colonization on *B. elliptica* infected lily leaves, 31 leaves (19 leaves from *U. atrum* treated plots and 12 leaves from water + 0.01% Tween 80 treated plots) with one newly formed lesion were selected. The development of the lesion and related tissue types was monitored. The tissue types distinguished were identical to those distinguished in the laboratory experiment on substrate specificity. Length and width of specific tissue types as well as of the whole leaf was measured at alternating 3 and 4 day intervals and the surface area for each tissue type was calculated assuming a rectangular shape for leaf, lesion and yellow streaks. Leaves with one lesion represent the best case scenario for the antagonist since multiple lesions per leaf would cause a much quicker colonization of the leaf by the pathogen with a strongly reduced formation of senescing and necrotic tissue in yellow streaks.

Lily climate chamber experiments

The effect of *U. atrum* on the expansion of lesions caused by *B. elliptica* was studied at a range of constant temperatures in mist chambers. Mist chambers were constructed from Lexan (General Electric, Pittsfield, Massachusetts), and measured 1.00 x 0.63 x 1.00 m (L x W x H). Mist chambers had an open bottom and a Lexan door and were placed on the perforated shelf of a climate chamber (Bioclim 1600 SP/+5..45 DU, Weiss Technik, Reiskirchen, Germany). The climate chamber allowed control of light periods (intensity and duration), temperature and relative humidity of the air

(RH) outside the mist chamber. Mist was produced by an ultrasonic humidifier (Stulz Ultrasonic FN 400 H, Stulz, Hamburg, Germany) and introduced into the mist chamber through a tube, perforated every 3 cm, mounted to the ceiling of the mist chamber and covering the full length of the chamber. After mist periods two ventilators mounted in the back wall of the mist chamber were used to ventilate the chamber, reducing the RH within the chamber to the set RH of the surrounding climate chamber. Timing and duration of mist - and ventilation periods and mist density were computer controlled. (A.J.A. van der Zalm and H.W. Roelofsen, unpubl.).

Plant material, inoculation and growing conditions

Bulbs of Asiatic hybrid lilies (*Lilium*) cv. Mont Blanc were grown for 12 weeks in pots in a greenhouse as described for the laboratory experiment on substrate specificity. No infections by *Botrytis* spp. or other diseases occurred on the leaves. Ten labelled leaves per plant were inoculated with a droplet of a *B. elliptica* conidial suspension (3µL with $5x10^4$ conidia/ml on the abaxial side of the leaf (Doss et al., 1984)). After inoculation the plants were incubated in a mist chamber in the dark, at the appropriate temperature and 100% RH for 24 hours. Plants were allowed to dry after which they were sprayed with a conidial suspension of *U. atrum* ($1x10^6$ conidia/ml) or with sterile tap water containing 0.01% Tween 80 until run-off using an atomizer (Desaga, Heidelberg, Germany). Spray treatments resulted in average *U. atrum* conidial densities of 29 and 38 conidia/mm^2 on the adaxial and abaxial side of the leaves respectively. Plants were allowed to dry before they were returned to the mist chamber for the rest of the experiment. Possible effects of *U. atrum* and temperature on the *B. elliptica* lesion expansion rate were determined at: 6°C, 12°C, 15°C, 18°C and 21°C.

Experimental design

The experiment was conducted in two mist chambers, each of which was placed in a separate climate chamber. Each temperature setting was sequentially tested in each mist chamber leading to a randomized block design in which the temperature settings were assigned randomly to each mist chamber. A mist chamber contained two blocks, each block contained 6 plants to which the 2 spray treatments were assigned randomly in triplicate.

Observations

B. elliptica lesions typically have an elliptical shape. The length and width of all lesions larger than 1x1 mm of each plant was recorded at 1 day intervals, or at 2 day intervals at 6°C. Measurements were stopped when the lesion reached the edge the leaf. Length- and width expansion rates of each lesion were calculated as the slope of the regression line between time (X-axis), and length, or width (Y-axis). Non established and non expanding lesions were excluded from the analysis.

Statistics

For cyclamen greenhouse experiments 2 and 3, disease severity was calculated for each replicate (plot) as the average number of leaves per plant sporulating with *B. cinerea*.

Log-linear regression models, with replicate and treatment as classifying variables, were fitted to the data on disease severity. The model assumes that the variance of the data is proportional to Poisson variance. Models were fitted to the data using the method of quasi likelihood (MacCullagh and Nelder, 1989). Overall effects of replicate and treatment were assessed using *F*-tests for the ratio of the mean deviance for the particular effect and the mean residual deviance. In case *F*-tests were significant ($P < 0.05$), treatment means on the log-scale were separated using *t*-tests ($P = 0.05$).

The Area Under the Disease severity Progress Curve (AUDPC) was calculated as the area under the curve of disease severity (Y-axis) against time (X-axis) and analysed using analysis of variance (ANOVA).

B. elliptica severity in field experiment 1 was analysed separately for each observation date using ANOVA.

From the data obtained in lily field experiment 2, the average sporulation intensity (SPLACI) per leaf was calculated per plot and sampling position for each of the distinguished fungal genera. Angular transformed sporulation data were then analysed using ANOVA, testing for the main effects of starting date, spray treatment, position in the canopy and their interactions.

The average lesion length- and width growth rate per plant, calculated from the data obtained in the lily climate chamber experiment, was subjected to ANOVA testing for main effects of temperature, treatment and their interaction. The relationship of lesion length- and width growth rate to temperature was studied by fitting a second

order orthogonal polynomal within the ANOVA including tests of the interaction of the linear- and quadratic trend with treatment.
In case *F*-tests were significant ($P < 0.05$) in the ANOVA's mentioned above, LSD tests ($P = 0.05$) were used for testing pairwise differences between the means. All analysis were performed with the statistical package Genstat 5 (Numerical Algorithmus Group, Oxford, UK).

Table 4.1. Substrate specificity of *B. elliptica* (Be), *B. cinerea* (Bc) and *U. atrum* (Ua) for lily and cyclamen tissues. Three different non-colonized tissue types and two tissue types pre-colonized during 48 hours were inoculated with conidial suspensions of *B. elliptica* (lily), *B. cinerea* (cyclamen) and *U. atrum* (both lily and cyclamen). Presence of mycelium within the tissue was determined immuno-histologically after 48 hours incubation at 18°C in a moist chamber. Classification is based on observations on 16 - 24 sections per host, inoculant and tissue type combination.

Tissue type	Lily						Cyclamen					
	Water[1]		*B. elliptica*[1]		*U. atrum*[1]		Water[1]		*B. cinerea*[1]		*U. atrum*[1]	
	Be[2]	Ua[2]	Be[2]	Ua[2]	Be[2]	Ua[2]	Bc[2]	Ua[2]	Bc[2]	Ua[2]	Bc[2]	Ua[2]
Non-colonized												
Healthy	–	–	+/-	-	-	-	–	–	–	–	–	–
Senescing	–	–	+	-	-	-	–	–	–	–	–	–
necrotic	–	–	+	-	-	++	–	–	+	–	–	+
Pre-colonized												
necrotic + *U. atrum*	–	++	+/-	++	-	++	–	++	–	++	–	++
necrotic + *B. elliptica*	++	–	++	-	++	+/-	nd	nd	nd	nd	nd	nd
necrotic + *B. cinerea*	nd	nd	nd	nd	nd	nd	++	–	++	–	++	+/-

[1] = Inoculant.
[2] = Fungus detected.
– = No mycelium detected.
+/- = Trace of mycelium detected.
+ = Mycelium clearly present.
++ = Mycelium abundantly present.
nd = Not Done.

Results

Substrate specificity

Results on internal colonization of comparable lily- and cyclamen tissues show a clear difference in the capabilities of conidia of *B. elliptica* and *B. cinerea* to infect or saprophytically colonize different types of host plant tissue (Table 4.1). *B. elliptica* conidia infected healthy and senescing lily leaf tissue and initiated saprophytical colonization of necrotic lily leaf tissue. *B. cinerea* conidia could not infect healthy or senescing cyclamen leaf tissue and were only capable of initiating saprophytical colonization of necrotic cyclamen tissue. *U. atrum* conidia were only able to initiate saprophytical colonization of necrotic lily or cyclamen tissue. When faced with established mycelium of *U. atrum* in the substrate, *B. elliptica* was capable of establishing very low levels of mycelium in pre-colonized necrotic lily leaves whereas *B. cinerea* was excluded from necrotic cyclamen leaves pre-colonized by *U. atrum*. *U. atrum*, was capable of establishing very low levels of mycelial colonization in necrotic lily and cyclamen tissue pre-colonized by *B. elliptica* or *B. cinerea*.
Macroscopical observations on the same leaves prior to sectioning showed lesion formation on healthy and senescing lily leaves inoculated with *B. elliptica* conidia. Healthy and senescing cyclamen leaves inoculated with *B. cinerea* conidia remained symptomless. Sporulation was only found on necrotic cyclamen leaves pre-colonized by *B. cinerea*. Non sporulating external mycelium was found on necrotic lily leaves inoculated with *B. elliptica* conidia, irrespective whether they had been pre-colonized by *B. elliptica*, by *U. atrum*, or not. Non sporulating external mycelium was also found on necrotic cyclamen leaves pre-colonized by *B. cinerea* or *U. atrum* and or inoculated with *B. cinerea* conidia.
From these results it is clear that necrotic tissue is the only mutual substrate for *Botrytis* spp. and *U. atrum* in both lily and cyclamen crops.

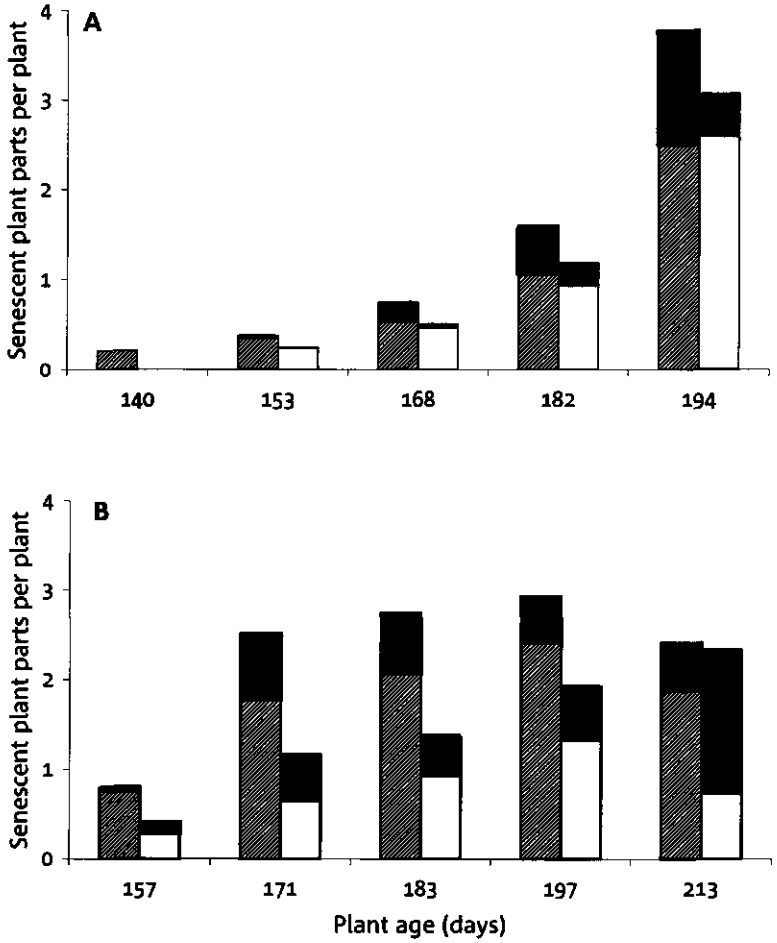

Figure 4.1. Average number of necrotic leaf blades (hatched bars) and necrotic petioles (white bars) per plant, sporulating (black top of bars) or non-sporulating (non black area of bars) with *B. cinerea*, in the commercial cyclamen crop of greenhouse experiment 1 (A) and the untreated plots of cyclamen greenhouse experiment 2 (B).

Cyclamen

Substrate availability

During greenhouse experiments 1 and 2 the number of green leaves of the cyclamen plants increased in a linear fashion from 22 to 65 and from 30 to 51 leaves per plant. Necrotic petioles and leaf blades, the mutual substrate for both *B. cinerea* and *U. atrum*, were available in increasing numbers from approximately 20 weeks after sowing (Fig. 4.1). During the experiments, sporulating *B. cinerea* was found on an increasing number of petioles and leaf blades but only after necrotic leaves had been observed (experiments 1 and 3) indicating that the presence of necrotic tissue is essential for infection of cyclamen plants by *B. cinerea*.

Biocontrol experiments

In experiments 2 and 3 regular removal of senescing, visually non - *B. cinerea*-infected, leaves was a highly effective control measure against *B. cinerea* (Table 4.2; Fig. 4.2). It is therefore highly likely that saprophytical colonization of necrotic tissue is an important step in the infection process of cyclamen by *B. cinerea*.
U. atrum applications resulted in a significantly lower disease severity and AUDPC value in experiment 3 as compared to both control treatments although *U. atrum* applications were not as effective against *B. cinerea* as the removal of senescing tissue. In experiment 2, where *B. cinerea* was present in the crop from the start, this effect was not pronounced. Nevertheless, the AUDPC value of both *U. atrum* treatments was significantly lower than the AUDPC value of the control treatment sprayed with water + 0.01% Tween 80 (Table 4.2, Fig. 4.2).

Lily

Substrate availability

At plant level in field experiment 1, on average 6 symptomless naturally necrotic leaves were present per plot of 150 plants on 27 May 1998. Their numbers increased to 80-100 leaves per plot in the first week of June after which it stabilised. Naturally necrotic leaves were mostly found in the lowest leaf layers of the canopy.

Table 4.2. Effect of *U. atrum* applications and the removal of senescing leaves on the Area Under the Disease severity Progress Curve (AUDPC) for *B. cinerea* severity in cyclamen greenhouse experiments 2 and 3.

Treatments	AUDPC	
Experiment 2		
Water + 0.01% Tween 80	91.7	a[1]
Not treated	76.4	ab
Removal of senescing leaves	4.0	c
U. atrum at 4 week intervals	56.5	b
U. atrum applied 1x	61.9	b
Experiment 3		
Water + 0.01% Tween 80	66.7	a
Not treated	76.6	a
Removal of senescing leaves	7.4	b
U. atrum at 4 week intervals	34.3	c

[1] Means of the same experiment followed by a common letter do not differ significantly ($P < 0.05$) according to ANOVA followed by an LSD test ($P = 0.05$).

After incubation, 20 - 50% of these leaves showed sporulation of *B. cinerea* whereas *B. elliptica* was not detected.

At leaf level in experiment 2, a small amount of necrotic tissue was available in the yellow streaks, mostly distally from the *B. elliptica* lesion. No indications were found that *U. atrum* treatments affected yellow streak formation on *B. elliptica* infected leaves in any way.

Necrotic tissue, the mutual substrate of *B. elliptica* and *U. atrum*, was therefore present during most of the growing season in the form of necrotic leaves in the lowest canopy layers and as part of the yellow streaks on *B. elliptica* infected leaves (Fig. 4.3).

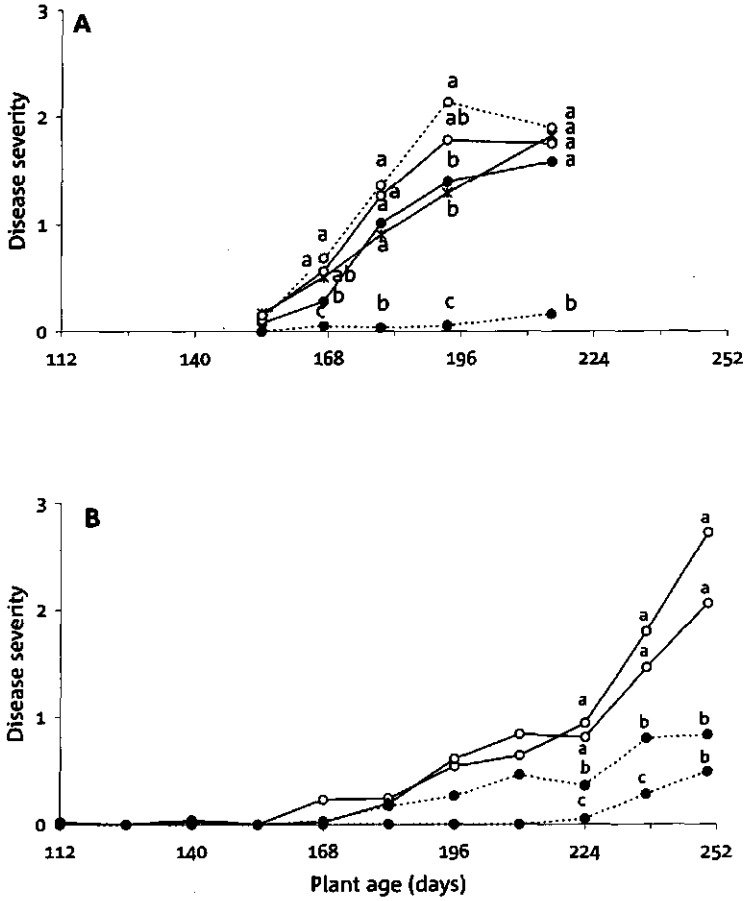

Figure 4.2. Effect of *U. atrum* applications and the removal of senescing, *B. cinerea* free, leaves on the disease severity of *Botrytis cinerea* in cyclamen cv Superserie in greenhouse experiments 2 (A) and 3 (B). Disease severity is defined as the average number of leaves with symptoms of *B. cinerea* per plant. (—○—): untreated control; (- -○- -): water containing 0.01% Tween 80 applied at 4 week intervals; (—●—): *U. atrum* ($1x10^6$ conidia/ml) applied at 4 week intervals; (—*—): *U. atrum* ($1x10^6$ conidia/ml) applied once, coinciding with the first treatment of the other spray treatments; (- -●- -): senescing (*B. cinerea* free) leaves removed at 3 - 4 day intervals. Data of experiment 3 were analysed starting at a plant age of 224 days. Values from the same assessment date with a common letter do not differ significantly ($P < 0.05$) according to log-lineair regression followed by *t* - tests.

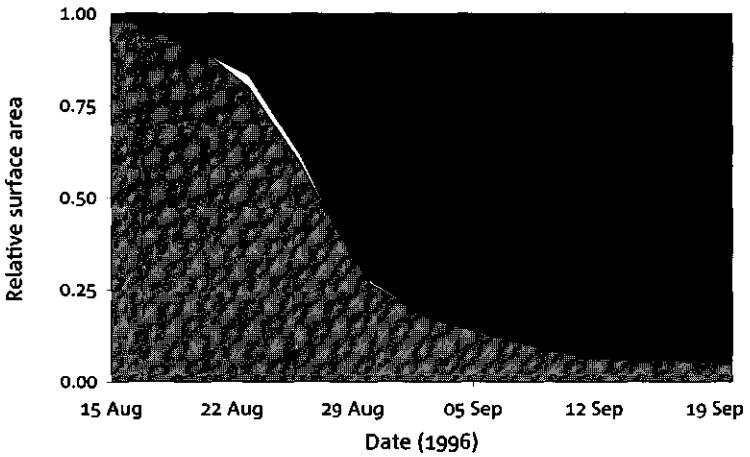

Figure 4.3. Relative surface area of different tissue types (substrates) present within *B. elliptica* infected lily leaves during a "fire" epidemic in lily field experiment 2. 31 leaves with a total area of 222 cm^2 were monitored during the growing season. (■) = B. elliptica lesion; (■) = healthy or senescing tissue; (□) = necrotic tissue.

Biocontrol experiment

At plant level in experiment 1, the disease severity was statistically the same for both treatments on all observation dates. Removal of the naturally necrotic tissue did thus not affect the epidemic of *B. elliptica* and necrotic tissue is therefore not part of an infection pathway of *B. elliptica* in lily.

At leaf level in experiment 2 it was commonly observed that *B. elliptica* lesions almost completely covered the leaf and that the pathogen was sporulating within the lesions at a low intensity. *Ulocladium* spp. and other saprophytes were usually found densely sporulating on small areas, usually the tip and or base of the leaf, outside the *B. elliptica* lesions. Regular *U. atrum* applications resulted in a significantly increased sporulation intensity (SPLACI) for *Ulocladium* spp. and a significantly reduced sporulation intensity of *B. elliptica* and *Cladosporium* spp. on necrotic lily leaves (Table 4.3). Other saprophytes were present (mainly *Alternaria* spp.) but their abundance was too low to be included in the analysis. No effect of the starting date of spray treatments on the sporulation of *B. elliptica* and *U. atrum* was found. *Cladosporium* spp. were significantly less abundant in plots within the main plot assigned to starting date 2. This effect was most likely caused by natural fluctuations

Table 4.3. Effect of *U. atrum* applications as compared to an untreated control on the sporulation (SPLACI) of *B. elliptica*, *Ulocladium* spp. and *Cladosporium* spp. on lily leaves infected by *B. elliptica* in lily field experiment 2.

Effect		SPLACI (%)[1]		
		B. elliptica	*Ulocladium* spp.	*Cladosporium* spp.
Starting date				
Starting date 1		10.9	4.4	8.5 a
Starting date 2		10.3	4.0	5.1 b
Treatment				
U. atrum		8.3 a	13.8 a	2.7 [2]
Control		12.7 b	0.1 b	9.9 [2]
Position				
High		14.8 a	5.8 a	3.6 [2]
Low		6.7 b	2.7 b	8.3 [2]
Treatment	**Position**			
U. atrum	High	12.6	17.7	0.7 a
	Low	4.8	10.3	5.9 b
Control	High	17.1	0.3	8.6 bc
	Low	8.9	0.0	11.0 c

ANOVA and comparison of means were done on angular transformed sporulation data. Means in the table are backtransformed to normal scale. Means in the same column for the same effect followed by a common letter do not differ significantly ($P = 0.05$). Means in the same column for the same effect not followed by any letter indicate a non-significant effect in the F - Test.

[1] SPLACI (%) is defined as the relative leaf area per leaf sporulating at maximum intensity.

[2] Means of individual effects are not separated due to a significant Treatment * Position interaction.

in the presence of *Cladosporium* spp. within the experimental field. *B. elliptica* and *U. atrum* were significantly more abundant in the highest leaf layers of the lily crop than in the lower leaf layers. At both levels in the canopy, *U. atrum* applications had a similar effect on *B. elliptica* as indicated by the absence of a Treatment * Position interaction for these two species. *Cladosporium* spp. were found equally abundant in the highest and lowest leaf layers in the control treatment. *U. atrum* applications were significantly more suppressive to the sporulation of *Cladosporium* spp. in the higher leaf layers then in the lower leaf layers. Thus *U. atrum* is colonizing necrotic

lily leaf tissue suppressing both, *B. elliptica* and naturally occurring saprophytes such as *Cladosporium* spp.

Climate chamber experiment

B. elliptica lesion length- and width growth rates increase linearly with temperature. *B. elliptica* lesions expand approximately twice as fast in the longitudinal direction as in the transversal direction.
At all temperatures tested *U. atrum* treatment did not significantly affect the length - or width growth rate of *B. elliptica* lesions as compared to the control treatment (Table 4.4). *U. atrum* did also not influence the establishment of lesions by *B. elliptica* as for both treatments the number of established lesions did not differ significantly.

Table 4.4. Effect of temperature on the length - and width growth rate of lesions caused by *B. elliptica* and linear and quadratic trends between temperature and the lesion growth rates as determined on lily leaves cv. Mont Blanc in the climate chamber experiment.

Temperature (°C)	*B. elliptica* lesion growth rate (mm/day)	
	Length growth rate	Width growth rate
6	4.3	1.4
12	5.2	2.6
15	7.9	3.4
18	7.2	3.9
21	9.1	5.2
Linear trend	0.32[1] (0.012[2])	0.24[1] (0.014[2])
Quadratic trend	0.04[3] (0.789[2])	0.006[3] (0.660[2])

[1] Linear increase of growth rate (mm/day) per unit of temperature (°C).
[2] *P*-value.
[3] Curvature.

Discussion

Comparative methods were used to explain differences in the effectiveness of *U. atrum* 385 as a biocontrol agent against two *Botrytis* species in different crops. *B. elliptica* in lily could not be controlled by *U. atrum* 385 applications (Köhl, unpubl.) whereas *B. cinerea* in cyclamen was successfully controlled by *U. atrum* 385 under commercial greenhouse conditions (Köhl et al., 1998). Substrate specificity in conjunction with life cycle characteristics of pathogen and antagonist were shown to play a decisive role in the potential effectiveness of *U. atrum* as a biocontrol agent against *Botrytis* spp. Necrotic tissue was identified as the key substrate for biological control of *Botrytis* spp. by *U. atrum*. Necrotic tissue is present in both crops during most of the growing season (Fig. 4.1, 4.3). The role of necrotic tissue in the epidemiology of *B. cinerea* in cyclamen and *B. elliptica* in lily was however markedly different. In cyclamen, saprophytical colonization of necrotic tissue, initiated by conidia, is an essential step in the infection of cyclamen plants by *B. cinerea*. Colonization of necrotic tissue by *U. atrum* excludes *B. cinerea* and disrupts the infection pathway (Table 4.1, Fig. 4.2). *U. atrum* applications against *B. cinerea* in cyclamen were less effective in greenhouse experiment 2 than in greenhouse experiment 3. This difference in effectiveness, which is in contrast with earlier findings (Köhl et al., 1998), is most likely caused by the difference in *B. cinerea* severity when *U. atrum* was first applied. In experiment 2 the established *B. cinerea* population could have dispersed through mycelial contact infections to healthy cyclamen petioles without a possibility for the antagonist to interfere. In experiment 3, *B. cinerea* was not found until well after the first *U. atrum* applications.
In lily, necrotic tissue is not part of an infection pathway of *B. elliptica*. Conidia of *B. elliptica* directly infect healthy lily tissue (Table 4.2) and bypass the pathway which could be effectively blocked by *U. atrum*. Nevertheless, *U. atrum* did partly colonize the necrotic tissue available on *B. elliptica* infected lily leaves and reduced *B. elliptica* sporulation under field conditions (Table 4.3). Based on spatial separation of sporulation of *B. elliptica* and saprophytic fungi and the absence of an effect of *U. atrum* on *B. elliptica* lesion expansion rates it is concluded that *U. atrum* is colonizing necrotic leaf tissue outside the lesion rather than interfering with the pathogen inside the lesion. Apparently, competitive exclusion is the dominant mechanism over co-colonization of tissue despite the fact that the antagonist is not necessarily excluded from *B. elliptica* colonized tissue (Table 4.1). A similar spatial separation was found in the interaction between *Pyrenophora tritici-repentis* and *Septoria*

nodorum (Adee et al., 1990) indicating this might be a common phenomena in fungal ecology.

Competitive exclusion is most likely based on competition for resources (Köhl et al., 1997). Therefore, the inability of *U. atrum* to co-colonize *B. elliptica* lesions cannot be generalized. Other host - pathogen combinations providing substrates with a different nutritional status, might well be more favourable to the antagonist. *U. atrum* applications to established *B. cinerea* lesions on bean leaves (*Phaseolus vulgaris*) reduced both lesion expansion and *B. cinerea* sporulation (Elad et al., 1994b). Other antagonists, with different nutritional requirements, might not be excluded from the *Botrytis* lesion. An additional mechanism of antagonism, such as antibiosis, might lead to sporulation reduction as described for the *Cochliobolus sativus* and *Trichoderma harzianum* interaction in wheat seedling leaves (Biles and Hill. 1988). *U. atrum* does not produce such metabolites (Köhl et al., 1997).

U. atrum was found to be more suppressive towards *B. elliptica* and naturally occurring saprophytes in the higher leaf layers of the lily canopy than in the lower leaf layers (Table 4.3). Elmer and Köhl (1998) contributed this effect to lower *U. atrum* conidial densities in the lower leaf layers. *U. atrum* conidial densities were not checked in the present experiment but since we used the application method of Elmer and Köhl (1998), the reduced antagonistic effect in low leaf layers is likely to have the same cause.

Despite the antagonistic effect of *U. atrum* applications on *B. elliptica* sporulation there were no indications that disease severity was affected. Apparently, the level of sporulation suppression (approximately 30% fewer spores produced, Table 4.3) was not enough to significantly affect the epidemic. Multiple infections of leaves, common in practice, rapidly expanding lesions and leaf to leaf contact infections, which contribute to dispersal of *B. elliptica* within and between plants, may be able to compensate for a 30% loss of conidia. Also, sporulation suppression as measured after the epidemic might have overestimated sporulation suppression during the epidemic. Furthermore, *U. atrum* could only successfully compete with the pathogen after *B. elliptica* has established lesions and directly or indirectly killed leaf tissue outside the lesions. Only then a small fraction of the leaves becomes available for colonization by saprophytic fungi, including *U. atrum* (Fig. 4.3).

B. cinerea is a ubiquitous plant pathogen with a host plant range of over 200 species (Jarvis 1977 ; 1980a). Nevertheless, *B. cinerea* was found to be a less aggressive pathogen than *B. elliptica*. This lack in aggressiveness was exploited by *U. atrum*, which interacted with the pathogen during its weakest phase, the colonization of necrotic tissue. Competition during the colonization of *B. cinerea* lesions is

presumably also taking place but here the pathogen has a competitive advantage (Table 4.1). *U. atrum* in necrotic cyclamen tissue also benefits from long interaction times, the absence of an escape for the pathogen (e.g. infection of healthy tissue) and protection from adverse abiotic conditions (Fokkema, 1993; Köhl and Fokkema, 1994). The apparent weakness of *B. cinerea* in cyclamen was also reported by Schlösser (1978) who found it was almost impossible to infect young cyclamen leaves using agar discs overgrown with *B. cinerea* mycelium in direct contact with the leaf. The presence of a 'saprophytical route' towards infection of healthy plant parts was also reported for *B. cinerea* in lettuce (Wood 1951), tomatoes (Newhook, 1957), grapes (Dubos et al., 1982) and for *Sclerotinia sclerotiorum* in bean (Boland and Hunter, 1988). *U. atrum* could thus be especially effective in such pathosystems. Recently, *U. atrum* has indeed been shown to effectively control *B. cinerea* in grapes under field conditions (Schoene and Köhl, 1999).

The present study has identified key factors for biological control of *Botrytis* spp. by *U. atrum*. This knowledge allows a quick assessment of the potential of the antagonist in different pathosystems. Substrate specificity of the fungi involved determines whether pathogen and antagonist come into direct contact. The competitive abilities of the antagonist determine the level of suppression of the pathogen in mutual substrates. The biocontrol effect is finally determined by both the role of the mutual substrate in the epidemiology of the pathogen and the competitive abilities of the antagonist in the mutual substrate. This rational approach identifies the target(s) and the aim for antagonist application, increases knowledge of the pathosystem and allows better timing of actual applications.

As a crop protection agent, *U. atrum* is taking a position different from agents that directly affect the pathogen's parasitic fitness (MacKenzie, 1978), such as fungicides and biocontrol agents effective through hyperparasitism or secondary metabolites. *U. atrum* accelerates the degradation of necrotic tissue which is essential for the pathogen as a stepping stone towards infection of the cyclamen plant. *U. atrum* therefore biologically controls *B. cinerea* in cyclamen through bio-sanitation, similar to - but more effective than - the natural saprophytic population.

Chapter 5

Biological control of *Botrytis cinerea* by the saprophytic antagonist *Ulocladium atrum*, a simulation study

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Abstract

CompFun, a spatially explicit dynamic simulation model describing saprophytic colonization of necrotic cyclamen leaf tissue by the plant pathogenic fungus *Botrytis cinerea* and the saprophytic fungal antagonist *Ulocladium atrum* was constructed and implemented within a two dimensional grid. Both fungi explore the leaf and utilize the resources in it. Fungal growth within grid cells is modelled using a Lotka-Volterra competition approach. Spatial expansion from grid cell to grid cell is assumed proportional to the biomass gradient between donor and receptor cell. Established fungal biomass is assumed to be immobile. Hence, only biomass influx into the grid cells is allowed and outfluxes are not included in the model. Radial growth rates of *B. cinerea* and *U. atrum* in necrotic cyclamen tissue were measured to determine parameters describing the spatial dynamics of the fungi. At most temperatures, *B. cinerea* colonies expanded twice as rapidly as *U. atrum* colonies. The model is used to study the competitive interaction between *B. cinerea* and *U. atrum* in cyclamen in relation to the biocontrol effect on *B. cinerea*. Spatial aspects of substrate colonization were well simulated but verification of temporal aspects of substrate colonization was hindered by substantial variation in the experimental data. Identification of the cause of this variation is essential for a better understanding of the system. The process of establishment of fungal mycelia also needs future attention. A sensitivity analysis demonstrated the importance of covering necrotic leaves with a uniform and dense cover of vital *U. atrum* conidia to maximize its control effect.

Introduction

Necrotrophic fungi of the genus *Botrytis* cause economically important diseases in many greenhouse and field crops. Recently, it has been established that the saprophytic fungal antagonist *Ulocladium atrum* Preuss isolate 385 holds promise as an effective biological control agent against *Botrytis* spp. (Schoene and Köhl 1999; Köhl et al., 1998, 1995b; Chapter 4). The biocontrol effect of *U. atrum* against *Botrytis* spp. is based on pre-emptive or competitive colonization of necrotic plant tissue. The competition affects colonization as well as sporulation of *Botrytis* spp. (Chapter 3). Suppression of sporulation of *Botrytis* spp. due to application of *U. atrum* has been observed in several *Botrytis* spp. - host combinations (Köhl et al., 1995b, 1995c; Chapter 3). Reduction of sporulation has the potential to slow down and delay or

even prevent epidemics of *Botrytis* spp. (Köhl et al., 1995a). In cyclamen (*Cyclamen persicum*) saprophytic colonization of necrotic tissue by *B. cinerea* is an essential step towards mycelial infection of healthy plant parts. Colonization of necrotic tissue by *U. atrum* excludes *B. cinerea* and blocks this infection pathway (Chapter 4). Repeated applications of *U. atrum* conidial suspensions to commercial cyclamen crops are equally effective against *B. cinerea* as fungicide applications (Köhl et al., 1998).
In this chapter, the model of Chapter 3 is further developed to take account of spatial phenomena. The study of spatial phenomena, like fungal colonization of a leaf from a single starting point, may yield different and relevant insights in the biological control abilities and requirements of *U. atrum*.
Such insights can guide selection of potential antagonists, target diseases and application strategies. Systems analysis and dynamic simulation models are useful tools to explore biological control strategies (Rabbinge et al., 1989) and have been applied successfully in assessing the potential of microbial biocontrol agents (Hau and Kranz, 1978; Knudsen and Hudler, 1987).
The model developed in this chapter simulates the spatio-temporal colonization of necrotic leaf tissue by *B. cinerea* and *U. atrum* as influenced by the density and distribution of conidia on the leaf and the presence of competing mycelium (colonies) in the leaf.
The model integrates basic information presented in Chapters 3 and 4 which was collected using the techniques described in Chapter 2. Model results are compared to experimental results and a sensitivity analysis of the model is performed.

Materials and Methods

Model concepts and implementation

The biological system

The model describes fungal growth and competitive phenomena in a necrotic cyclamen leaf which is saprophytically colonized by *U. atrum* and *B. cinerea*. Fungal

colonies originate from conidia which in the case of *U. atrum* are introduced through spray applications while the conidia of the pathogen result from natural spore production and dispersal. Conidia germinate and establish a mycelial micro-colony or die. Mycelia grow through extension and branching of hyphae which explore and utilize the substrate (Rayner and Webber, 1984). Established colonies expand radially and merge at contact if they are of the same species. Contact between colonies of *B. cinerea* and *U. atrum* results in a deadlock for *B. cinerea* whereas *U. atrum* is capable of a low level of co-colonization within a *B. cinerea* colony (Chapter 3). Interaction between the mycelia of *Botrytis* spp. and *U. atrum* affects the growth rate of the mycelia but it does not result in any negative effect on existing biomass. The dominating antagonistic mechanism is nutrient competition (Köhl et al., 1997; Chapter 3). Both fungi colonize the necrotic leaf and compete for a shared and limiting amount of primary resources (Chapter 3). Depletion of the resources stops mycelial growth. Secondary resources i.e. resources left over by a preceding colonizer (Cooke and Rayner, 1984) are not utilized by *B. cinerea* and only to a limited extent by *U. atrum* but this has no known effect on the pathogen (Chapter 3). Sporulation develops from established mycelia.

Model development

CompFun is a spatially explicit dynamic simulation model of competitive colonization of necrotic cyclamen leaf tissue by *B. cinerea* and *U. atrum*. Processes are modelled within the two dimensional environment of a grid representing the leaf. Grid cells are the smallest spatial units present in the model and state variables are assumed to be homogeneously distributed within grid cells. The state variables within CompFun are conidial densities of *B. cinerea* and *U. atrum* on the leaf and mycelial biomass densities of both fungi within the leaf. Conidia germinate on the leaf and give rise to an initial biomass density in the leaf at the time germination is complete. Biomass in the leaf grows within grid cells and spreads from grid cell to grid cell. Growth of biomass is limited by the availability of a shared resource. Resource differentiation between both fungi as reported in Chapter 3 is not taken into account because secondary resource capture by *U. atrum* has no known negative short term

influence on *B. cinerea*. It also does not have any known consequences for *U. atrum*. Data presented in Chapter 3 suggest that after establishment of mycelial biomass resources are reallocated from the mycelium to the conidia resulting in degradation of the mycelium. These aspects are not included in the model.
The basic assumptions of the model are as follows: 1) Resource uptake by the mycelium is local and is only used for growth while maintenance respiration is negligible. 2) The rate of resource uptake is proportional to the growth rate and independent of mycelial age. 3) Resource competition is assumed to be the only mode of interaction between both fungal species. 4) Fungal mycelia do not die during the simulation period.

The grid A necrotic cyclamen leaf was represented by a leaf shaped grid consisting of square cells of 0.5 x 0.5 mm. Simulations were done with whole leaves (11.5 cm^2) or half leaves (6.1 cm^2). Grid cell size was chosen at the same scale as mycelial biomass (0.7 x 0.7 mm) and conidial density were determined (0.5 x 0.5 mm or 1 x 1 mm). Furthermore, grid cell length had to be at least as large as the distance covered by the fastest expanding fungus in one time step (0.004 mm/minute for *B. cinerea* at 24°C, Fig. 5.3). The model was implemented using PCRaster, a GIS based software package which allows dynamic modelling in a spatially variable environment (Department of Physical Geography, Utrecht University, the Netherlands). In PCRaster a stack of PCRaster maps (grids) represents the necrotic leaf. The model includes a map for each of the state variables. All processes only take place within the borders of the leaf.

Germination of conidia Germination of conidia on the leaf was simulated using the fixed boxcar train technique (Goudriaan and Van Roermund, 1993). This technique keeps track of all successive development stages of conidia during the germination process. Average germination time of *B. cinerea* and *U. atrum* conidia was 6.6 and 5.2 hours respectively at 18°C. Variation in development time among individual spores, 26% for *B. cinerea* (Yunis et al., 1994) and 22% for *U. atrum* (Köhl et al., 1999), was implemented using boxcar trains with 15 and 21 boxcars respectively.

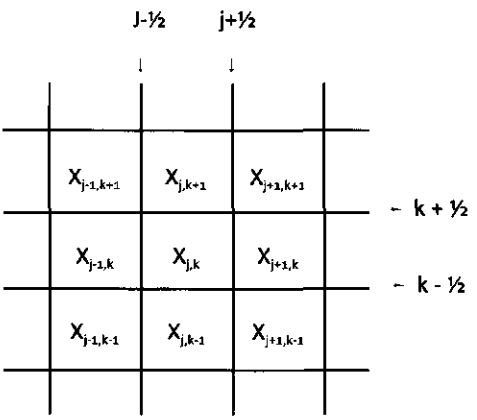


Figure 5.1. Schematic representation of a grid representing variable "X" and the coordinate system used to designate grid cells and borders between grid cells.

Growth of fungal biomass

The mycelial biomass in grid cells increases through a combination of growth within the grid cell (local growth) and invasive contributions (spatial expansion) from neighbouring grid cells. Growth is limited by the availability of nutrients, but the latter are not explicitly present in the model. Instead, local growth was simulated using a logistic growth model in which resource capture is made explicit in the state variables representing the biomass of the competitors. This choice was made to eliminate unnecessary complexities from the model.

Logistic growth equations are implemented as follows: Cell (j,k) is the grid cell with j and k as x- and y- coordinates for the cell centre (Fig. 5.1). Let $B^t_{j,k}$ be the biomass density of *B. cinerea* of cell j,k at time t. $U^t_{j,k}$ represents the *U. atrum* biomass density of cell (j,k) at time t. The biomass growth rate for *B. cinerea* and *U. atrum* due to local growth and not considering competition is then given by:

$$\frac{dB_{j,k}}{dt} = r_b B_{j,k}\left(1 - \frac{B_{j,k}}{B_{max}}\right) \qquad \text{(equation 5.1)}$$

$$\frac{dU_{j,k}}{dt} = r_u U_{j,k}\left(1 - \frac{U_{j,k}}{U_{max}}\right) \qquad \text{(equation 5.2)}$$

Here, $B_{j,k}$ is the biomass density of *Botrytis* in grid cell (j,k). Biomass is expressed as a volume fraction because this is the way in which the substrate occupancy was quantified (Chapter 2, 3). Likewise, the maximum colonization level K_b is expressed as a volume fraction. r_b is the relative growth rate of local growth of *Botrytis* (time^{-1}). Estimates for the relative growth rates of fungal biomass (r_b and r_u) and the initial biomass assigned to germinated conidia (I_b, I_u) were calculated from the values listed in Table 3.2. Straight lines were fitted to log-transformed mycelial colonization data obtained in experiment 1 of Chapter 3. Only data from the first 60 hours of (near exponential) biomass increase were included in this analysis. The relative growth rates (r_b and r_u) were estimated as the slope of the fitted lines at 18°C. The initial biomass of a germinated conidium was also estimated from the regression line. The total biomass generated after the average germination time (given above) was back transformed and divided by the conidial density to yield I_b and I_u.
The maximum colonization levels of both fungi (K_b and K_u) were estimated as the average maximum colonization level in Fig. 3.2A - 3.2D. Table 5.1 lists all variables and parameters included in the model.

Spatial expansion occurs in the x and y direction through each of the four boundaries of a grid cell (Fig. 5.1). The flux (F) of fungal biomass from grid cell (j,k) to (j+1,k) through boundary J+½ (Fig. 5.1) is proportional to the biomass gradient between the grid cells. For *B. cinerea* this results in:

$$Fb_{j+½} = -S_b \frac{B_{j+1,k} - B_{j,k}}{\Delta x} \Phi b_{j+½} \qquad \text{(equation 5.3)}$$

S_b is a dispersal coefficient and Δx is the grid cell width. Equation 5.3 resembles Fick's law for diffusive flux but fungal mycelium is immobile. Mycelium grows into neighbouring grid cells through hyphal elongation without biomass being removed from the donor grid cell. Therefore, inflow of biomass in a receptor grid cell does not result in outflow of biomass from the donor grid cell. Consequently, only influx of biomass is considered. A flux limiter, $\Phi b_{j+½}$ with values of either 0 or 1, is used to prevent outflux but allow influx. The flux limiter functions as a switch. It is also used

to prevent biomass dispersal from grid cells with a biomass density too low for spatial expansion. This biomass dispersal threshold density is implemented as a fraction τ_b of the maximum colonization level K_b.
For *B. cinerea* the flux limiter is defined as:

$$\Phi b_{j+\frac{1}{2}} = \begin{cases} 0, & B_{j+1,k} < B_{j,k} \\ 0, & B_{j+1,k} < \tau_b K_b \\ 1, & \text{otherwise} \end{cases} \qquad \text{(equation 5.4)}$$

Analogous equations and flux limiters were used for biomass fluxes through the other three boundaries. The total *B. cinerea* biomass influx potential into grid cell (j,k) then equals the sum of the four influxes:

$$\frac{1}{\Delta t}\left(B_{j,k}^{t+1} - B_{j,k}^{t}\right) = \frac{-1}{\Delta x}\left(Fb_{j+\frac{1}{2},k}^{t} - Fb_{j-\frac{1}{2},k}^{t}\right) + \frac{-1}{\Delta y}\left(Fb_{j,k+\frac{1}{2}}^{t} - Fb_{j,k-\frac{1}{2}}^{t}\right) \qquad \text{(equation 5.5)}$$

Combining equation 5.3 and analogous equations for *B. cinerea* biomass influx potential and equation 5.5 and taking into account that $\Delta y = \Delta x$ results in the total influx potential of *B. cinerea* into grid cell (j,k):

$$B_{j,k}^{t+1} - B_{j,k}^{t} = S_b \frac{\Delta t}{\Delta x^2}\left[\Phi b_{j+\frac{1}{2}}\left(B_{j+1,k}^{t} - B_{j,k}^{t}\right) + \Phi b_{j-\frac{1}{2}}\left(B_{j-1,k}^{t} - B_{j,k}^{t}\right) + \Phi b_{k+\frac{1}{2}}\left(B_{j,k+1}^{t} - B_{j,k}^{t}\right) + \Phi b_{k-\frac{1}{2}}\left(B_{j,k-1}^{t} - B_{j,k}^{t}\right)\right]$$

(equation 5.6)

Table 5.1. Variables, parameters and their estimates at 18°C included in the CompFun model.

	Symbol	estimate	Unit
Variables			
Conidial density	B_{con}, U_{con}		conidia/mm^2
Biomass density	B, U		mycelial volume fraction in grid cell [-]
Parameters			
Establishment parameters:			
Germination rate	V_b	0.0025	[minute^{-1}]
	V_u	0.0033	
Initial mycelial biomass of a germinated conidium	I_b	0.000018	mycelial volume fraction in grid cell [-]
	I_u	0.000042	
Logistic growth parameters:			
Relative growth rate	r_b	0.0012	[minute^{-1}]
	r_u	0.0010	
Maximum colonization level	K_b	0.05	mycelial volume fraction in grid cell [-]
	K_u	0.05	
Spatial parameters:			
Dispersal coefficient	S_b	0.01398	mm^2 minute^{-1}
	S_u	0.00459	
Dispersal threshold	τ_b	0.85	mycelial volume fraction in grid cell [-]
	τ_u	0.85	

An analogous set of equations was developed for *U. atrum*.

Estimates for the dispersal coefficients (S_b and S_u) were obtained through calibration with experimental observations on radial colony expansion in necrotic cyclamen leaves (described below). The dispersal thresholds (τ_b and τ_u) determine the shape of the mycelial fronts as they are seen in a radial cross section through the colony.

Values close to zero result in a long tail of low biomass levels preceding the main body of the colony, whereas values close to one result in a steep mycelial front. Quantitative data to estimate the dispersal threshold were not available but histological observations invariably show a steep mycelial front for both *B. cinerea* and *U. atrum*. The mycelial density at the edge of the colony declines from the maximum colonization level within the colony to zero outside the colony within 0.5 - 2 mm. The dispersal thresholds (τ_b and τ_u) were arbitrarily set to 0.85 to match those histological observations.

Competition for resources is assumed to take place within grid cells and is modelled using an approach previously used by Lotka (1925), Volterra (1931) and Zegerman (1987). As implemented here, both species are experiencing interspecific competition in exactly the same way as intraspecific competition through the depletion of a single shared resource by the two species of fungi. The competitive effect of both species is expressed in a competition coefficient α which reflects the resource use efficiency. α is indexed as α_{ub} to characterize the effect of *U. atrum* on *B. cinerea* and vice versa for α_{bu}. The first of these coefficients is calculated as K_u/K_b while the other is calculated as K_b/K_u (Table 5.1).

Overall, the growth potential of *B. cinerea* biomass in grid cell (j,k) is given by the potential for local growth plus the biomass influx potential. Both potentials for growth are however limited by the resources available within the grid cell (j,k). Thus equation 5.1 for local growth and equation 5.6 for total influx potential can be combined into an equation describing the *B. cinerea* biomass increase in grid cell (j,k) accounting for local growth, biomass influx and resource limitation:

$$B_{j,k}^{t+1} = \left(r_b B_{j,k} + \textit{influx potential B. cinerea} \right) \left(1 - \frac{B_{j,k} + \alpha_{bu} U_{j,k}}{B_{max}} \right) \qquad \text{(equation 5.7)}$$

An analogous equation was derived for *U. atrum:*

$$U_{j,k}^{t+1} = \left(r_u \, U_{j,k} + \textit{influx potential U. atrum} \right) \left(1 - \frac{U_{j,k} + \alpha_{ub} \, B_{j,k}}{U_{max}} \right) \qquad \text{(equation 5.8)}$$

Time step The time step was based on germination of *U. atrum* conidia, the fastest process included in the model. The average germination time of *U. atrum* conidia is 2.8 hours at 30°C which is the optimal temperature (Köhl et al., 1999). Implementation of the germination process in a fixed boxcar train of 21 boxcars leads to a residence time of 8 minutes per boxcar. Hence, a time step of 1 minute was deemed appropriate.

Output PCRaster stores maps of *B. cinerea* and *U. atrum* biomass as well as classified maps showing the presence of both species in a single map after each requested time interval. The leaf area occupied by *U. atrum*, by *B. cinerea* and by both fungi was calculated and stored in tables. After a simulation run the output maps can be viewed, one by one or in animation, and they are available for further processing. Variables, stored in tables, can be exported for further processing or viewed as graphs.

Parameterization of the spatial spread of *B. cinerea* and *U.atrum*

Estimates for the dispersal coefficients S_b and S_u were derived by calibration on radial growth rates which were measured after point inoculation of both fungi in monoculture on sterile necrotic cyclamen leaves. Leaf tips were point inoculated using a sterile glass stamp (Chapter 3) and incubated in the dark at constant temperatures: 5, 12, 18, 24 and 30°C for *B. cinerea* and 5, 12, 18, 24, 28 and 33°C for *U. atrum*. Seven replicate petri dishes with two leaves each were included for each species - temperature combination. Two replicate experiments were carried out. Successful inoculations resulted in clearly visible, brown, radially growing colonies.

Histological studies confirmed that the brown area corresponded to internally colonized leaf tissue. The distance travelled by the front of the colony was measured daily along a marked transect using a ruler.

The radial growth rate was calculated as the slope of the linear regression of measured colony radius (Y-axis) on time (X-axis). To characterize the temperature response of radial growth rate, Logan curves (Logan et al., 1976) were used. Logan curves are widely used in insect biology to describe temperature dependent rate phenomena and have the following form:

$$Y_T = \psi \left[e^{(\rho (T - T_b))} - e^{\left(\rho (T_M - T_b) - \frac{(T_M - T)}{\Delta T}\right)} \right] \qquad \text{equation 5.9}$$

These curves generally describe an assymetric sigmoid increase of the response variable growth rate (Y_T) with temperature to an optimum temperature, after which the growth rate quickly decreases to zero at the maximum temperature T_M. The base temperature T_b was set to 5°C for both fungi. The other parameters were determined by non linear least squares regression using Genstat 5 (Numerical Algorithms Group Inc., Oxford, UK). The parameters are interpreted as follows: ψ is approximately equal to the radial growth rate at the base temperature. ρ characterizes the curvature of the increase of Y_T towards the optimum temperature. ΔT represents the temperature range over which Y_T declines from the maximum at the optimum temperature to zero at the maximum temperature.

Validation

Two types of validation were carried out. The first focuses on spatial phenomena and uses newly collected experimental data on colony development in cyclamen leaves. The second type of validation focuses on temporal aspects of model performance. For these validations, time series of fungal density are used which were measured microscopically in necrotic cyclamen leaves (Fig. 3.2D). Moreover, the ability of the model to reproduce the head start experiments, described in Chapter 3, is tested.

These experiments explore how different (relative) times of inoculation with conidia of *B. cinerea* or *U. atrum* affect the ultimate sporulation intensity of both fungi on necrotic cyclamen leaf. Experimental results and simulated results were reproduced in drawings or graphs and compared visually.

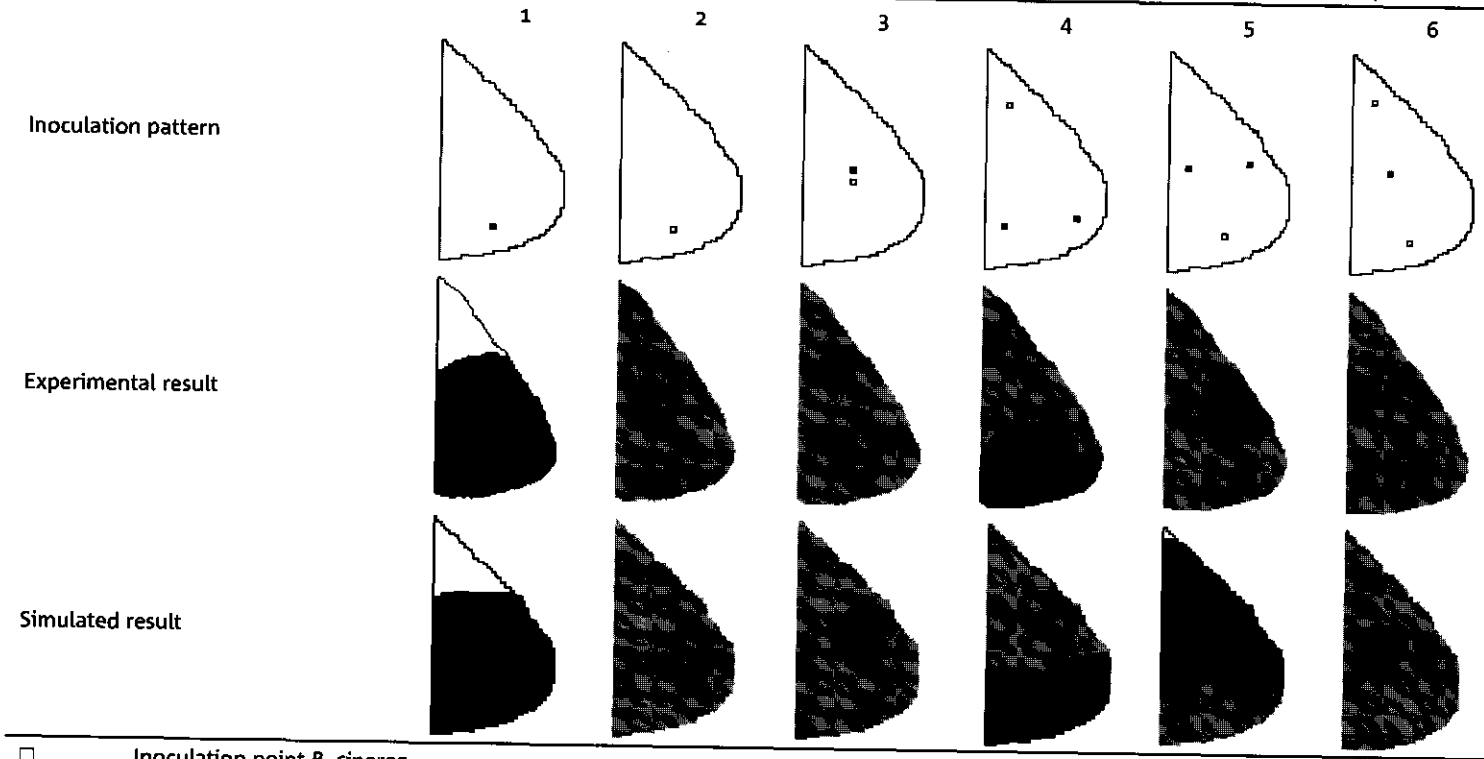
Spatial aspects

Colonies of *B. cinerea* and *U. atrum* were started in different spatial arrangements on necrotic half sterile cyclamen leaves. Leaves were point inoculated with conidia (described in detail in Chapter 3). Twelve different inoculation patterns were used (Fig. 5.2). The leaves were incubated in the dark at 18°C . Inoculations resulted in 66 ± 43 (standard deviation) and 127 ± 80 conidia per inoculation point for *B. cinerea* and *U. atrum* respectively. Four replicate leaves were included for each inoculation pattern. After two weeks incubation the sporulation patterns developed on the leaves were reproduced in a drawing and the number of successful inoculations and the surface area occupied by each of the fungi was recorded.

The experimental inoculation patterns of Fig. 5.2 were reproduced in the simulations. All spores per inoculation point were deposited in a single 0.5 x 0.5 mm^2 grid cell. The exact number of conidia per inoculation point was randomly drawn from a lognormal distribution with parameters corresponding to the average and standard deviation of the logarithm of the number of conidia per inoculation point in the experiment (Oude Voshaar, 1994). Spore number was rounded to the nearest integer. Simulations for each of 4 replicate leaves were included. Simulation time was two weeks for each run. The resulting colonization patterns were reproduced in Fig. 5.2.

Drawings of experimental results and CompFun result maps were compared visually. The total colonized leaf area and the leaf area colonized per established colony of *B. cinerea* and *U. atrum* were calculated for each replicate. Colonized leaf area per fungus per leaf and leaf area colonized per colony were subjected to analyses of variance (ANOVA) testing for differences between experiments and simulation results.

Figure 5.2. Colonization of sterile necrotic halved cyclamen leaves after point inoculation with *Botrytis cinerea* and/or *Ulocladium atrum*. Inoculation pattern and experimental and simulated results after two weeks incubation at 18°C. Depicted leaves were randomly selcted from four replicate leaves.



□ Inoculation point *B. cinerea*.

■ Inoculation point *U. atrum*.

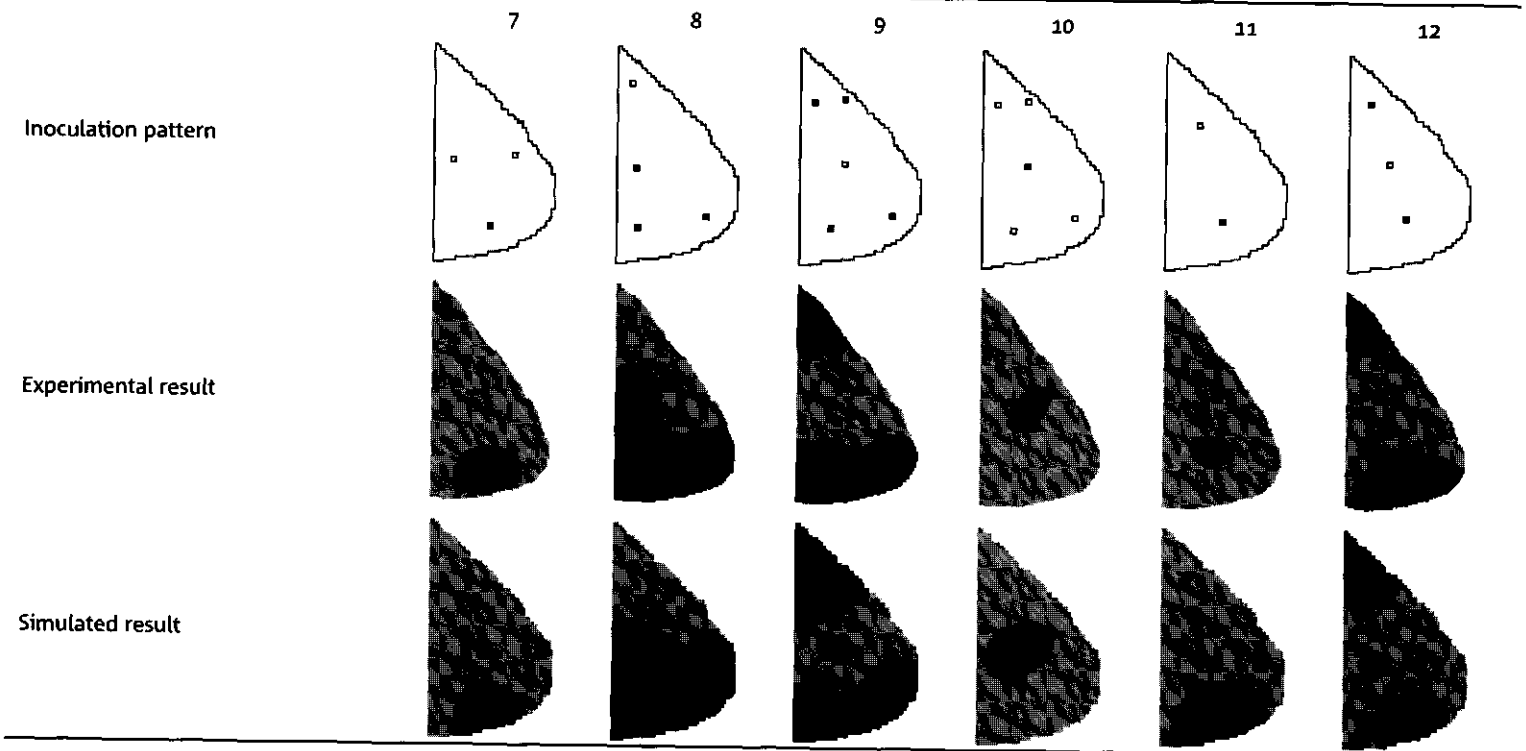
Light grey: Experimental: leaf area sporulation with *B. cinerea* only. Simulated: leaf area colonized by *B. cinerea* mycelium only.

Black: Experimental: leaf area sporulation with *U. atrum* only. Simulated: leaf area colonized by *U. atrum* mycelium only.

Dark grey Simulated leaf area with mycelial colonization of both *B. cinerea* and *U. atrum*.

White Uncolonized leaf area.

Figure 5.2. Continued



□ Inoculation point *B. cinerea*.

■ Inoculation point *U. atrum*.

Light grey: Experimental: leaf area sporulation with *B. cinerea* only. Simulated: leaf area colonized by *B. cinerea* mycelium only.

Black: Experimental: leaf area sporulation with *U. atrum* only. Simulated: leaf area colonized by *U. atrum* mycelium only.

Dark grey: Simulated leaf area with mycelial colonization of both *B. cinerea* and *U. atrum*.

White: Uncolonized leaf area.

Differences between inoculation patterns and the interaction between these factors were also tested. In case *F*-tests were significant ($P < 0.05$), LSD tests ($P = 0.05$) were used to test pairwise differences between the means. All analyses were done using Genstat 5. Colonized leaf area was expressed as a proportion of total leaf area and angularly transformed before analysis. The colonized leaf area per colony was only included in the analysis for leaves where all inoculation points resulted in established colonies.

Temporal aspects

In Chapter 3, a series of experiments is described in which the internal colonization of necrotic cyclamen leaf is followed through time, using the immuno-histological techniques of Chapter 2. The last of these experiments (Fig. 3.2D) was used for model validation. In this experiment, conidia of *B. cinerea* and *U. atrum* were homogeneously inoculated on sterile necrotic cyclamen leaves. The average conidial density was 25 ± 6 mm^{-2} for *B. cinerea* and 35 ± 5 mm^{-2} for *U. atrum*. The standard deviations given are the population parameters, and describe the variability among different 1 mm^2 areas. Three types of inoculations were included: *B. cinerea* alone, *U. atrum* alone, and *B. cinerea* and *U. atrum* together. The internal mycelial colonization level was determined at regular intervals during incubation. Five replicate leaves were included for each combination of treatment and sampling time. Further details are given in Chapter 3.
In the simulations, conidial densities per grid cell were randomly drawn from a lognormal distribution and rounded to the nearest integer. The parameters of the lognormal distribution corresponded to the average and standard deviation of the logarithm of the conidial density per grid cell in the experiment. As in the experiment, five replicate leaves were included in the simulation. Simulation time was two weeks for each run. Average colonization levels were calculated per leaf after each time step and graphically compared to the experimental results.

The second experiment used for validating temporal aspects of model performance was the "head start experiment" described in Chapter 3. This experiment aimed at

measuring the effect of differences in the time of inoculation of the conidia of *B. cinerea* and *U. atrum* on the final sporulation by both species, following homogenous inoculation of sterile necrotic cyclamen leaves with one or both fungal species. Conidial densities were 46 ± 16 mm^{-2} for *B. cinerea* and 45 ± 9 mm^{-2} for *U. atrum*. Application intervals between both inoculants created competitive advantages for one or the other species. Sporulation intensity was assessed after two weeks incubation at 18°C. Analysis focused on the relative spore 'yields', in a fashion similar to the way de Wit (1960) analysed experiments in plant competition with replacement series. Details are given in Chapter 3.
Simulations were carried out , using the same initial conditions (variable conidial densities, 10 application intervals), temperature and growing period of two weeks as in the experiment. A difference between experiment and simulation in this case is that the experimental data are based on sporulation intensities, whereas the simulation results are hyphal biomass densities. Simulations were compared graphically to the experimental data.

Sensitivity analysis

A sensitivity analysis was performed by modifying separate parameters (Table 5.1) with +20% or -20%. Because such large parameter changes gave deviant results for the dispersal threshold, this parameter was modified with +5% or -5%. The relative sensitivity of the output variable to these perturbations was calculated as $(\Delta Z/Z)/(\Delta Y/Y)$ where Z is the relevant output variable and Y is the parameter (Rossing et al., 1989). Two initial situations were distinguished: point inoculations and homogeneous inoculations, and for each spatial inoculation pattern mono- and mixed cultures of *B. cinerea* and *U. atrum* were considered. Therefore, the sensitivity analysis comes in four parts. Whole cyclamen leaves were used as substrate in the simulations. Patterns 1 and 11 (Fig. 5.2) were used for point inoculations. To test the sensitivity for germination parameters, 10 conidia were placed in a single grid cell representing the inoculation point. To test logistic growth parameters, the simulations were started with the grid cells representing the inoculation points filled up to the maximum colonization level with fungal biomass. Spray inoculations were

implemented by depositing 10 conidia in each grid cell (equivalent to 40 conidia mm^{-2}).

In each of the four situations chosen for sensitivity analysis, an output variable was selected that is sensitive to the changes in parameter values. For single species point inoculations the output parameter was the surface area of the colony just before it reached the edges of the leaf. For two species point inoculations, the output variable was the surface area covered by *B. cinerea* and *U. atrum* after the two weeks simulation time. Any area co-colonized by *B. cinerea* and *U. atrum* was divided evenly over both species. For single species homogeneous inoculations, the average colonization level after 100 hours was used as output variable. For two species homogeneous inoculations the average colonization level after two weeks simulation time was used as output variable.

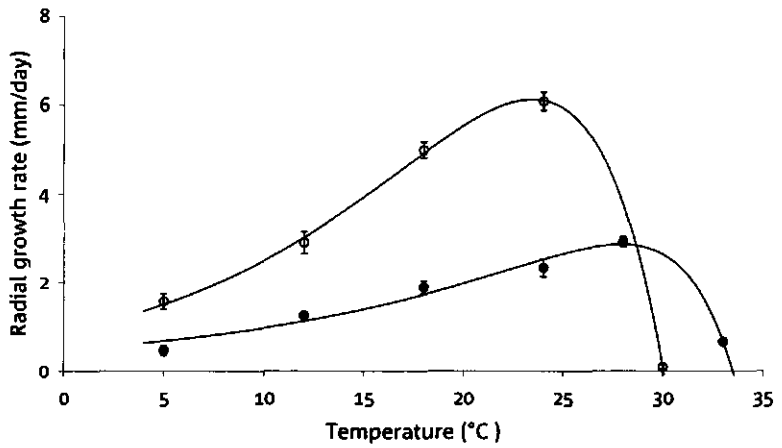


Figure 5.3. Effect of temperature on the radial growth rate of *Botrytis cinerea* and *Ulocladium atrum* colonies growing in sterile necrotic cyclamen leaf tissue. (○) = *B. cinerea*. (●) = *U. atrum*. (——) = Logan curves fitted to the experimental data. Error bars represent the standard error of the mean over two experiments.

Results

Parameterization of the spatial spread of *B. cinerea* and *U.atrum*

The radius of experimental colonies growing in sterile necrotic cyclamen increased linearly with time. The response of the experimental radial growth rate of *B. cinerea* and *U. atrum* colonies growing in sterile necrotic cyclamen leaves to temperature was well described by the fitted logan curves (Fig. 5.3). Parameter estimates (with standard error in brackets) for the Logan curves were as follows: *B. cinerea*: ψ = 1.85 (0.31) mm day^{-1}, ρ = 0.12 (0.01)°C^{-1}, T_M = 30.0 (0.03) °C , ΔT = 5.3 (0.5) °C, T_b = 5°C. *U. atrum*: ψ = 0.67 (0.20) mm day^{-1}, ρ = 0.07 (0.03) °C^{-1}, T_M = 33.5 (0.04) °C, ΔT = 2.8 (2.5) °C, T_b = 5 °C. The % variance accounted for was 99.6 and 93.1 for *B. cinerea* and *U. atrum* respectively. The radial growth rate at 18°C was calculated to be 4.9 mm day^{-1} for *B. cinerea* and 1.7 mm day^{-1} for *U. atrum*. The dispersal coefficients S_b and S_u were calibrated to 0.01398 for *B. cinerea* and 0.00459 for *U. atrum* at 18°C.

General behaviour of the model

Simulated point inoculations resulted in circular colonies, radially growing at a constant radial growth rate. Mycelial biomass in the colony reached the maximum colonization level in the centre with a steep mycelial front at the edges of the colony. Competitive interactions after point inoculations resulted in small areas of overlap between colonies of both species (Fig. 5.2).

Validation

Spatial aspects

CompFun result maps and experimental results are represented in Fig. 5.2 for one randomly chosen replicate leaf for each experimental and simulated inoculation pattern. In general, the simulated and experimental colonization patterns are very

similar indicating that the trends concerning spatial aspects of colonization are well captured by the model.

Analysis of variance on the proportion of leaf area colonized by *B. cinerea* and *U. atrum* resulted in significant differences between experiments and simulations and in some instances showed an effect of inoculation pattern. The interaction between both effects was not significant. On average 71.3% of the leaf was colonized by *B. cinerea* in experiments while 61.2% colonization was reached in simulations. For *U. atrum* the experimental and simulated colonized area's were respectively 21.6% and 34.5%. Thus the simulations overestimate the area covered by *U. atrum* while they underestimate the area covered by *B. cinerea*. This difference is due to an overestimation of the establishment of *U. atrum* colonies in the simulations. Experimentally, 78% of the *U. atrum* inoculations were successful whereas *B. cinerea* inoculations were 100% successful. Simulated inoculations were successful by default because establishment was not included in the model as a separate process. The effect of inoculation pattern on the proportion of leaf area colonized per species originated from the effect of the different spatial arrangements of the inoculation patterns. Inoculations in the centre of the leaf offer a better opportunity for the fungi to capture leaf area then inoculations in a confined area such as the leaf tip.

Analysis of variance on the relative leaf area colonized per colony resulted in a significant interaction between the effect of the origin of the data (experimental versus simulated) and inoculation pattern. The comparison of means is given in Table 5.2. The average size of an individual colony decreased with increasing numbers of inoculation points. For eight of the twelve inoculation patterns (1-4, 6, 8, 10 and 12) the simulated colonization patterns were very similar to the experimental patterns while there was no significant difference in the area covered per colony. The most notable exception was pattern 5. This pattern resulted in highly variable results in both simulations and experiments. When *U. atrum* established quickly and closed the gap between both inoculation points, *B. cinerea* was excluded from the top part of the leaf resulting in a colonization pattern resembling the simulated pattern. When *U. atrum* did not close the gap in time, *B. cinerea* was given access to the top part of the leaf, resulting in a colonization pattern resembling the experimental pattern

Table 5.2. Comparison of experimental and simulated colonization patterns. Experimental and simulated Leaf area covered per colony of *B. cinerea* and *U. atrum* following different patterns of point inoculation (Fig. 5.2) and two weeks incubation at 18°C.

Inoculation pattern (Fig. 5.2)	Average surface area per colony (% of leaf surface)									
	B. cinerea					*U. atrum*				
	Experimental		Simulated			Experimental		Simulated		
1	0[1]	a	0	a		79.0	a	79.8	a	
2	100	b	100	b		0	b	0	b	
3	98.5	bc	93.4	cd		2.7	c	6.6	cd	
4	61.4	efgh	49.8	efg		23.8	ef	25.1	ef	
5	89.6	cdj	54.8	efg	*[2]	9.1	cdgh	21.1	efjk	*
6	48.3	efg	43.2	ef		2.3	c	12.9	cdghjkk	
7	45.8	ef	46.3	ef		16.8	ehjk	6.6	cd	*
8	61.0	efg	34.6	ek		16.4	ehjk	21.8	efk	
9	74.0	hl	49.2	efg	*	6.7	cdg	12.6	cdghjk	
10	23.2	k	23.0	k		7.2	cdg	7.4	cdg	
11	94.3	cd	67.9	gh	*	5.7	cd	32.1	f	*
12	85.7	djl	77.3	hjl		14.3	eghjk	11.3	cdghj	

[1] Comparison of means after analysis of variance on angular transformed average colony area. Means in the table are backtransformed to the original scale. Means of the same species followed by a common letter do not differ significantly at $P = 0.05$ according to an LSD test.

[2] * : The experimental and simulated colonization pattern are significantly different.

given in Fig. 5.2. Significant differences between simulated and experimental leaf area per colony were detected for patterns 7, 9 and 11. In pattern 11, the simulated *U. atrum* colony extended to the edge of the leaf while in the observations, *U. atrum* appeared as sporulating islands, entirely surrounded by *B. cinerea*. This may be caused by a difference in the exact position of the inoculation point. For patterns 7 and 9, the simulated colonization patterns were very similar to the experimental colonization patterns despite a significant difference detected between the simulated and experimental leaf area per colony. In all instances except pattern 7, the difference between the simulated and experimental results indicate that the

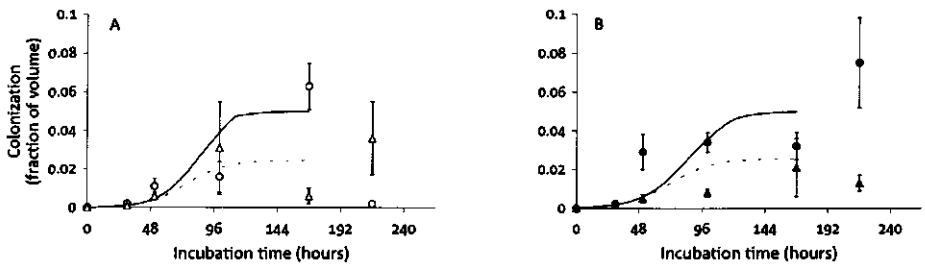


Figure 5.4. Time course of colonization of sterile necrotic cyclamen leaf tissue by *Botrytis cinerea* (A) and *Ulocladium atrum* (B) after spray inoculation with conidial suspensions ($1x10^6$ conidia per ml) of either or both fungi at time = 0. Experimental and simulated results. (○) = experimental *B. cinerea* monoculture. (●) experimental *U. atrum* monoculture. (Δ) = experimental *B. cinerea* in mixed culture with *U. atrum*. (▲) = experimental *U. atrum* in mixed culture with *B. cinerea*. (——) = Simulated result of the monoculture. (- - - -) = Simulated result of the mixed culture. Error bars represent the standard error of the experimental means of five replicate leaves.

simulated colony expansion of *U. atrum* overestimated the experimental colony expansion whereas that of *B. cinerea* underestimated reality. The simulated results for pattern 7 underestimated the *U. atrum* colony expansion (Table 5.2) whereas the *B. cinerea* colony expansion was simulated correctly.

For pattern 3, *B. cinerea* and *U. atrum* were inoculated very close to each other. This created an unstable initial interaction in which either species was able to surround the colony of the other and claim the vast majority of the substrate area. Differences in initial conidial densities on the inoculation site were responsible for this effect.

Non spatial aspects

Results of the experimental and simulated temporal colonization experiments are given in Fig. 5.4. Although the general characteristics of the experimental temporal colonization patterns are captured, the large variation within and between sampling times in the experimental data is not reproduced by the model. The causes for the variability in the experimental observations are unknown.

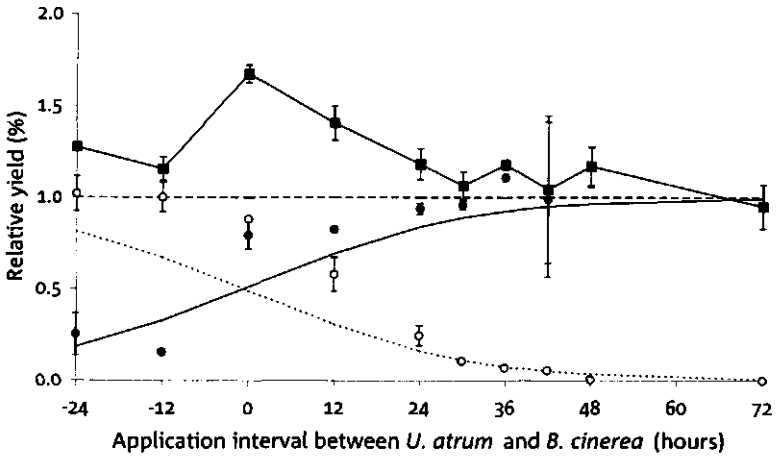


Figure 5.5. Relative yields (RY, average yield in mixed culture divided by the average yield in the corresponding monoculture) and relative yield totals (RYT) of an experimental and simulated head start experiment. Sterile necrotic cyclamen leaf tissue was competitively colonized by *Botrytis cinerea* and *Ulocladium atrum* after spray inoculation with conidial suspensions ($1x10^6$ conidia/ml) of *B. cinerea* and *U. atrum*, *B. cinerea* and sterile tap water or *U. atrum* and sterile tap water at different application intervals ranging from 24 hours application advantage for *B. cinerea* (-24 hours) to 72 hours application advantage for *U. atrum* (72 hours). (○) = *B. cinerea* experimental RY. (●) *U. atrum* experimental RY. (—■—) = experimental RYT . (——) = *U. atrum* simulated RY. (- - - -) = *B. cinerea* simulated RY. (— — —) = simulated RYT. Experimental RY's and RYT are based on sporulation. Simulated RY's and RYT are based on the mycelial colonization level of leaves. Error bars represent standard error of the mean over two experiments.

Results from the experimental and simulated head start experiment are given in Fig. 5.5. The general shape of the experimental RY lines was reproduced by the simulated RY lines. Quantitatively however, the simulated RY lines for *B. cinerea* and *U. atrum* were less steep than the experimental RY lines and the intersection of both simulated RY lines is displaced downward and sideways when compared to the experimental RY lines. The simulated RYT line does not display the typical convex shape of the experimental RYT line.

Sensitivity analysis

Point inoculated monocultures

Colony surface area responds to changes in all parameters except the maximum colonization level of *B. cinerea* or *U. atrum* (Table 5.3A). Increasing the establishment parameters (l_b, l_u, V_b and V_u) or relative growth rates (r_b and r_u) resulted in an increased colony surface area. Increasing the dispersal parameters resulted in an increased colony surface area for the dispersal coefficients (S_b and S_u) and a strongly decreased colony surface area for the dispersal thresholds (τ_b and τ_u). Colony surface area appeared highly sensitive to the parameters determining spatial expansion, especially to the dispersal threshold. Changes in the establishment parameters (l_b, l_u, V_b and V_u) and logistic parameters (r_b, r_u, K_b and K_u) have much less, but still significant, effect on the colony surface area.

Point inoculated mixed cultures

The proportion of leaf area colonized by *B. cinerea* or *U. atrum* responded in a similar manner to parameter changes as the individual colony size in single species simulations described in the previous section (Table 5.3B). The trends described for point inoculated monocultures remain valid for point inoculated mixed cultures. *U. atrum* is more sensitive to changes in the parameters than *B. cinerea*, probably due to its smaller radial growth rate.

Uniformly inoculated monocultures

In contrast to the point inoculated situations, the system is totally insensitive to changes in the spatial parameters (Table 5.3C). From the logistic and establishment parameters, the relative growth rates (r_b and r_u) had the largest influence on the average colonization level of the leaf after 100 hours. Quantitatively, *B. cinerea* and *U. atrum* responsed similarly to changes in model parameters.

Table 5.3. Sensitivity analysis of the model CompFun. The effect of six parameters for *U. atrum* and for *B.cinerea*, is investigated in four initial situations on whole cyclamen leaves: point inoculation in monoculture (pattern equivalent to pattern 1, Fig.5.2); point inoculation in mixed culture (pattern equivalent to pattern 11, Fig. 5.2); uniform inoculation in monoculture; uniform inoculation in mixed culture. Four each situation a sensitive output variable was chosen: colony surface area, colonized leaf area, colonization level after 100 hours and final colonization level respectively. Relative sensitivity is calculated as the ratio of percentage change in the output variable and the percentage change in the parameter. Parameters are the relative rate of local growth (r_b, r_u), maximum colonization level (K_b, K_u), dispersal constant (S_b, S_u), dispersal threshold (τ_b, τ_u), germination rate (V_b, V_u), and initial biomass of a pre-established colony (I_b, I_u).

Parameter	Reference value	Unit	Relative sensitivity			
			U. atrum		*B. cinerea*	
			+	-	+	-
A: Point inoculated Monoculture[1]						
V_b	0.0025	minute^{-1}			0.21	0.15
I_b	0.000018	[-]			0.36	0.37
r_b	0.0012	minute^{-1}			0.20	0.29
S_b	0.01398	mm^2 minute^{-1}			1.28	1.31
K_b	0.05	[-]			0.00	0.00
τ_b	0.85	[-]			-8.09	-7.82
V_u	0.0033	minute^{-1}	0.15	0.38		
I_u	0.000042	[-]	0.61	0.77		
r_u	0.0010	minute^{-1}	0.50	0.37		
K_u	0.05	[-]	0.00	0.00		
S_u	0.00459	mm^2 minute^{-1}	1.59	1.28		
τ_u	0.85	[-]	-7.32	-8.83		
B: Point inoculated dual culture[1]						
V_b	0.0025	minute^{-1}	-0.06	-0.07	0.01	0.01
I_b	0.000018	[-]	-0.19	-0.18	0.04	0.03
r_b	0.0012	minute^{-1}	-0.20	-0.22	0.04	0.05
K_b	0.05	[-]	0.00	0.00	0.00	0.00
S_b	0.01398	mm^2 minute^{-1}	-0.82	-1.23	0.17	0.26
τ_b	0.85	[-]	8.10	4.61	-1.72	-0.98
V_u	0.0033	minute^{-1}	0.03	0.08	-0.01	-0.01
I_u	0.000042	[-]	0.17	0.24	-0.03	-0.05
r_u	0.0010	minute^{-1}	0.29	0.31	-0.06	-0.07
K_u	0.05	[-]	0.00	0.00	0.00	0.00
S_u	0.00459	mm^2 minute^{-1}	0.91	0.93	-0.19	-0.20
τ_u	0.85	[-]	-5.43	-5.33	1.16	1.13

[1] Point inoculation with 10 conidia per inoculation point, 1 grid cell in size.

[2] Uniform inoculation with 10 conidia per grid cell.

Table 5.3. Continued

Parameter	Reference value	Unit	Relative sensitivity			
			U. atrum		*B. cinerea*	
			+	-	+	-
C. Uniformly inoculated monoculture[2]						
V_b	0.0025	minute^{-1}			0.09	0.14
I_b	0.000018	[-]			0.21	0.28
r_b	0.0012	minute^{-1}			1.08	2.03
K_b	0.05	[-]			0.72	0.80
S_b	0.01398	mm^2 minute^{-1}			0.00	0.00
τ_b	0.85	[-]			0.00	0.00
V_u	0.0033	minute^{-1}	0.07	0.10		
I_u	0.000042	[-]	0.25	0.32		
r_u	0.0010	minute^{-1}	1.16	2.03		
K_u	0.05	[-]	0.69	0.76		
S_u	0.00459	mm^2 minute^{-1}	0.00	0.00		
τ_u	0.85	[-]	0.00	0.00		
D. Uniformly inoculated mixed culture[2]						
V_b	0.0025	minute^{-1}	-0.17	-0.26	0.19	0.30
I_b	0.000018	[-]	-0.38	-0.49	0.43	0.55
r_b	0.0012	minute^{-1}	-0.28	-0.38	0.32	0.43
K_b	0.05	[-]	0.00	0.00	1.00	1.00
S_b	0.01398	mm^2 minute^{-1}	0.00	0.00	0.00	0.00
τ_b	0.85	[-]	0.00	0.00	0.00	0.00
V_u	0.0033	minute^{-1}	0.13	0.19	-0.15	-0.21
I_u	0.000042	[-]	0.47	0.57	-0.53	-0.64
r_u	0.0010	minute^{-1}	0.30	0.36	-0.34	-0.41
K_u	0.05	[-]	1.00	1.00	0.00	0.00
S_u	0.00459	mm^2 minute^{-1}	0.00	0.00	0.00	0.00
τ_u	0.85	[-]	0.00	0.00	0.00	0.00

[1] Point inoculation with 10 conidia per inoculation point, 1 grid cell in size.

[2] Uniform inoculation with 10 conidia per grid cell.

Uniformly inoculated mixed cultures

In general, the trends described for the uniformly inoculated monocultures remain valid for their mixed culture equivalent (Table 5.3D). Quantitatively, the changes in the average colonization level of the leaf after two weeks were not as large as the changes in the average colonization level of the leaf after 100 hours for single species inoculations described in the previous section.

Discussion

The purpose of the research presented in this chapter was to integrate knowledge about competitive substrate colonization of necrotic cycleamen leaf tissue by *B. cinerea* and *U. atrum*, presented in Chapter 3, with measurements of colony expansion. This resulted in CompFun, a model that represents the basic phenomena governing the competitive interaction and spread of these two competing fungi, which may be used to study options of biological control of *Botrytis*. The model captures the essence of the biological processes without going into too much biological detail. Including additional mechanistic biological detail would be very interesting, but would also require additional data. The current model version fully exploits the available information.

The integration level of the CompFun model is suitable to study practical as well as more fundamental questions. Models describing growth and morphogenises at the level of individual hyphae (e.g. Edelstein, 1982; Edelstein et al., 1983; Trinci, 1984; Wessels, 1988) would be unsuitable to study questions at leaf level or higher. Models including less detail than the present model, e.g. describing fungal colonies as radially growing circles (e.g. Van Oijen, 1989), cannot account for growing and competiting mycelia within the same space and might therefore be more limited in their usefulness in evaluating biological control options. A spatially explicit approach was chosen because colony expansion is under some circumstances a practically relevant process. This is primarily due to spatial heterogeneity in conidial densities on the leaf and mycelial presence in the leaf.

Within CompFun, resource uptake is strictly local, mycelial age does not influence resource uptake, maintenance respiration is absent and mycelia do not die. These assumptions are - in the given order - increasingly at odds with biological reality. Experimental data have shown that mycelia do die and even dissolve (Chapter 3), putatively caused by autolysis of cell walls, initiated in the ageing mycelium by nutrient deprivation (Wessels & Sietsema, 1984). Maintenance respiration may be negligable when compared to growth respiration but experimental data to check the credibility of this hypothesis are not available. Resource uptake is unlikely to be stricly local due to the nutrient transport capabilities of hyphae. Following uniform inoculation, nutrient gradients may not exist and resource uptake may then, at least in theory, be local. The impact of these factors thus also depends on the inoculation pattern.

Fungal growth and competition of two strains of *Fusarium* has previously been described using a similar Lotka-Volterra competition approach (Zegerman et al., 1987) as the one used in this paper. To the best of our knowledge this is the first time that logistic growth of fungal biomass and "Lotka-Volterra" competition have been combined in a spatially explicit model. Temporal aspects of substrate colonization were dominated by the relative growth rate of both fungi and the intial biomass per grid cell generated by germinating conidia (Table 5.3). Spatial dispersal was modelled using a flux-approach in which inflow of biomass was accounted for without the transported amount being substracted from its source. Flux of biomass, normally resulting in dispersal of biomass over space, was thus turned into a spatial growth process describing the biology of fungal growth. Implementation of a flux limiter was necessary to prevent the artifact of numerical dispersion (Goudriaan, 1973) resulting in long tails of low biomass density preceding the true mycelial front. The values assigned to the threshold values for spatial dispersal were chosen to match histological observations on the mycelial front. In doing so, the flux limiter - dispersal threshold combination was turned into a biological parameter whose value was higher than the values commonly assigned to flux limiters (typically 0.01 - 0.1) to prevent unrealistic spatial dispersal. Dispersal was dominated by the value of the dispersal threshold although the model result was also sensitive to the dispersal coefficient (Table 5.3). Spatial aspects of colony were understandably uninfluential when inoculations were uniform. For practical disease management this implies that high *U. atrum* conidial densities, as uniformly distributed over the leaf as possible,

will be most effective in colonizing newly available necrotic tissue and suppressing *B. cinerea*. Spray applications must then be applied as the phenology of necrotic tissues in the crop dictates. Autogenic conidial production by *U. atrum* in a crop is unlikely to be sufficient. Data on this subject are however not available. *B. cinerea* has a considerable competitive advantage due to its larger radial growth rate.

Spatial expansion was well described by the model. The relative leaf area colonized by *U. atrum* was overestimated. Average colony size on the other hand was mostly well simulated. Insufficient knowledge on establishment of colonies after conidial inoculation is the most likely cause of these differences. This process, which is also likely to be highly sensitive to adverse environmental conditions was not explicitely included in the model. It may be studied in more detail and included in the model to increase the applicability of the model.

The comparison of simulated and temporal trends of substrate colonization was complicated by large standard errors of the experimental results obtained using the immuno-histolocial techniques described in Chapter 2. The large errors point to large spatial variability of colonization in the substrate, which is not replicated in simulated colonization patterns, and to an insufficient number of replications in the experiments. Given the laboriousness of the immuno-histological techniques, it would be difficult to use higher numbers of replications. Future studies might focus on explaining the spatial variability of colonization in the leaf, which might be due to spatial heterogeneity in substrate quantity or quality. No significant differences between simulated and observed progress of colonization were found during the first ca 4 days (Fig. 5.5). However, the inaccuracy of the experimental data implies that even major discrepancies would not reach statistical significance. Hence, research into the patterns of spatial variability of fungal density in the substrate, and the causes for this variability, are necessary to make progress in the understanding of fungal ecology.

The discrepancy between experimental and simulated head start experiments underline the quantitative differences conidial reproduction and mycelial colonization. For a better understanding of the ecology of *Botrytis* spp. and *U. atrum* and for accurate predictions of the effect of antagonist application on the disease epidemic, the relationship between mycelial colonization and sporulation should be

investigated further. Nutritional factors are also likely affect this relationship (Engelkes et al., 1997).

Qualitative aspects of the dynamic interaction between *B. cinerea* and *U. atrum* in necrotic cylamen leaf are adequately captured by CompFun. Quantitative understanding of the system is hindered by unidentified, spatially variable factors determining fungal growth and development. Putatively one of them was identified as the nutritional composition of the substrate. The impact of this factor may be limited when it equally affects the pathogen and antagonist but differential nutritional compositions in different crops are likely. For fundamental as well as practical purposes identification and quantification of this factor or factors deserve future attention.

Model results indicate that the spatial interaction between *B. cinerea* and *U. atrum* in necrotic cyclamen leaves can be represented by a simpler model based on radially growing circles (Van Oijen, 1989). Such a model would not take account of the two fungi competing for resources at a single point in the substrate, but in situations arising from point inoculations, the spatial processes are much more important then local resource competition, which may in such a situation be safely eliminated from the model. For practical biocontrol purposes however, uniform conidial densities of *U. atrum* are recommended. A model including local resource competition is therefore relevant to explore biological control options. CompFun has the best of both worlds, including equations that capture both the temporal and spatial competition mechanisms between *B. cinerea* and *U. atrum*. CompFun therefore deserves to be upgraded bringing in more explanatory process knowledge. The upgraded model may be used to perform scenario studies and guide *U. atrum* applications under practical conditions. The model can be coupled with a crop level model that includes the phenology of necrotic substrates - in which the competition takes place - as well as the phenology of target substrates (flowers, fruits) that must be defended against *Botrytis* spp., and the mechanisms by which *Botrytis* spp. migrate from one substrate to the other. Success of biological control against *Botrytis* spp. requires that the competitive interactions on necrotic substrates are won by *U. atrum*, such that *Botrytis* spp. loose their bridgeheads on necrotic substrates, which are often essential and indispensible for the pathogen to infect valuable fruits and flowers.

Chapter 6

General discussion

General discussion

Plant pathogenic *Botrytis* spp. cause economically important diseases in a wide range of host species throughout the world (Coley-Smith et al., 1980). The control of *Botrytis* diseases is based on frequent fungicide applications but remains difficult due to the 'explosive' nature of the diseases and due to fungicide resistance frequently developed by *Botrytis* spp. (Gullino, 1992; Leroux, 1994). Increasing concerns about the effects of pesticides on the environment and human health lead to increasing restrictions on the use of pesticides (Jansma et al., 1993). Biological control of *Botrytis* spp. could offer an alternative or supplement to existing control measures against *Botrytis* spp. The saprophytic fungus *Ulocladium atrum* was shown to be an effective biological control agent of *B. cinerea* (Köhl et al., 1995b, 1998; Schoene and Köhl, 1999). The biocontrol effect of *U. atrum* against *Botrytis* spp. is based on a competitive interaction between both species in necrotic plant tissue (Köhl et al., 1997). The nature of the interaction between *Botrytis* spp. and *U. atrum* in necrotic plant tissue was further studied in the research described in this thesis. The aim was to elucidate key ecological principles underlying the biological control potential effect of *U. atrum* and to contribute to the development of effective and practical biocontrol strategies based on *U. atrum* against *Botrytis* spp.

Ecology of *B. cinerea* and *U. atrum*

Plant and crop level

Necrotic plant tissue was the only mutual niche for *Botrytis* spp. and *U. atrum* in the pathosystems *B. elliptica* - lily (*Lilium*) and *B. cinerea* - cyclamen (*Cyclamen persicum*). *U. atrum* can only be effective as a biocontrol agent if colonization of necrotic tissue by *U. atrum* affects the epidemiology of *Botrytis*. Ageing and dead leaves were identified as the primary target for *U. atrum* applications in cyclamen crops. If these leaves are saprophytically colonized by *B. cinerea*, they serve as a stepping stone towards mycelial infection of healthy leaves. As *B. cinerea* conidia are not able to infect healthy cyclamen tissue, *U. atrum* colonization of necrotic tissue blocks this mycelial infection pathway resulting in effective biocontrol of *B. cinerea*. In contrast, *B. elliptica* conidia can successfully infect healthy lily leaf tissue. Ageing and necrotic

lily tissues were no part of a *B. elliptica* infection pathway. Therefore, no effective biocontrol strategy for *B. elliptica* through *U. atrum* applications could be identified. It remains to be investigated whether *U. atrum* can be employed to reduce *B. elliptica* primary inoculum through a competitive interaction with *B. elliptica* in crop debris during winter.

Due to the nature of the competitive interaction between the mycelia of *B. cinerea* and *U. atrum*, curative effects of *U. atrum* applications may not be expected. The secondary colonization established by *U. atrum* within *B. cinerea* colonies did not directly affect the pathogen's reproduction. The production of consecutive crops of conidia by the pathogen may be affected but due to large quantities of conidia produced before secondary colonization is established and due to the polycyclic nature of *Botrytis* spp. epidemics, a significant effect on disease development is not expected.

Theoretically, *U. atrum* could affect any pathogen that relies on necrotic plant tissue in any part of the disease cycle. The present study has identified two key factors at plant or crop level that determine the potential of *U. atrum* as a biocontrol agent. 1) The prerequisite of a mutual niche for pathogen and *U. atrum*. 2) The role of the mutual substrate in the pathogen's disease cycle. The entire disease cycle has to be evaluated for these properties to assess the potential of *U. atrum* as a biocontrol agent.

Tissue level

The mycelia of *B. cinerea* and *U. atrum* exploit the resources available in necrotic plant tissue. Internal mycelium is relatively protected from adverse conditions in the phyllosphere and crucial for exploitation of resources and survival. Consequently, the spatio-temporal dynamics of mycelial colonization of necrotic tissue were our prime interest in studying the competitive relationship between *Botrytis* spp. and *U. atrum*. Mycelium growing on the leaf surface was not studied during the present project.

Development of *B. cinerea* and *U. atrum* biomass in necrotic cyclamen leaf tissue followed logistic patterns in monocultures as well as in mixed cultures. The individual maximum colonization and sporulation levels were lower in mixed culture than in monoculture. However, not all factors determining fungal growth and development were captured in the experiments. Nutritional factors (Engelkes et al., 1997) were put forward as the most likely responsible factor for the variation observed in the experimental data. In cyclamen, differential substrate quality did not

result in a competitive advantage for pathogen or antagonist. In other crops this is not necessarily true. The relative competitive abilities of pathogen and antagonist thus have to be determined for each new crop or substrate.
Competition for primary resources was identified as the dominant antagonistic mechanism between *B. cinerea* and *U. atrum*. Head start experiments demonstrated that the race for resources in necrotic cyclamen tissue was decided within 48 hours. Relatively simple nutrients, which can be readily metabolised, are therefore likely to constitute the majority of the key nutrients. Specific knowledge on which nutrients are essential for *Botrytis* spp. and *U. atrum* may offer an opportunity to manipulate the substrate to the advantage of the antagonist by adding specific nutrients. The decline of biomass observed in the latest phases of the colonization process may offer another opportunity to manipulate the pathosystem. Triggering of the lytic response of *B. cinerea* mycelium early in the colonization process or even before infection may offer a novel approach to disease control.

Systems analysis and simulation

CompFun, a spatially explicit dynamic simulation model describing the colonization of necrotic cyclamen leaves by *B. cinerea* and *U. atrum* was developed. CompFun integrates key ecological processes identified within the framework of this thesis. CompFun was used to study the competitive interaction between *B. cinerea* and *U. atrum* and to identify knowledge gaps. Adapted forms of the model can be used to study interactions between fungi in a wider context.

Spatial colonization patterns were well predicted by the model. Simulated temporal colonization patterns of fungal biomass captured the general behaviour of the system but did not generate the experimentally observed variation. Identification of the causes for this variation is fundamental to the understanding of the system and might offer new opportunities for disease control. Nutritional factors were postulated as a major cause for this variation. Key nutritional factors therefore have to be identified and included in the model. For practical biocontrol purposes, an approach similar to the approach used in Chapter 3, utilizing relative amounts of selected groups of nutrients, may be preferred. For more fundamental ecological questions, a detailed identification of the nutrients involved may be more appropriate. Identification of nutritional key factors will also facilitate a more

effective assessment of the possibilities of *U. atrum* as a biocontrol agent in other crops and against other pathogens.

Sporulation of *B. cinerea* and *U. atrum* was not included in the model. Sporulation is generated by the fungal mycelium but quantitative relationships between mycelial colonization and sporulation could not be established. Variability, paramount in observations on mycelial colonization, was reduced when the accompanying sporulation was considered. Apparently *B. cinerea* and *U. atrum* have the capability to safeguard their reproductive output despite poor substrate quality. Conidial quality might be affected by substrate quality but was not investigated in this project. However, conidia of *B. cinerea* drive the disease epidemic and a reduction in quantity or quality of the conidia might result in disease suppression. The relationship between mycelial colonization and sporulation thus needs further attention and may result in coupling of the present monocyclic population model at tissue level to epidemiological models at crop level.

Climatological aspects were not included in the model. Especially water availability and temperature are known to be determinative factors for fungal growth and development. The effect of these climatological factors on each of the model parameters should be investigated and included in CompFun if this model is to be developed further.
Crop phenology determines the availability of colonizable substrates for pathogen and antagonist and should be included in the model when CompFun is used for practical purposes.

Biological control of *Botrytis* spp.

U. atrum has the potential to become a successful, commercially available, biological control agent (Köhl, 1998; Schoene and Köhl, 1999). The mode of action of *U. atrum*, pre-emptive colonization, is however completely different from the mode of action of present day chemical control agents. This implies that timing of *U. atrum* applications should not be based on the pathogen's infection pressure but on crop phenology. As soon as the target necrotic tissue is formed, *U. atrum* can be effectively applied, independent of the presence of the pathogen. The effect of well timed *U. atrum* applications then depends on establishment of the antagonist and on disease pressure of the pathogen. The relationship between pathogen disease

pressure, antagonistic conidial density and related factors should be explored further and can be used to guide *U. atrum* applications and integrate a new biocontrol agent into existing crop protection strategies. Antagonist establishment deserves special attention. Advantages of *U. atrum* applications include a reduced chemical burden on the environment and human health and a reduced risk of resistance development by *Botrytis* spp.

The potential of *U. atrum* against *B. cinerea* in other crops and against other pathogens in other crops is largely unexplored. Schoene and Köhl (1999) successfully applied *U. atrum* against *B. cinerea* in grape and cyclamen. Due to the general nature of the antagonistic interaction it seems plausible that more pathosystems can be identified where *U. atrum* will successfully reduce diseases. The tools developed during the present project facilitate an effective selection of new pathosystems. Three aspects are of prime importance: 1) identification of the mutual niche or substrate of the pathogen and *U. atrum*, 2) establishment of the relative competitive abilities of the pathogen and *U. atrum* in their mutual substrate and 3) establishment of the role of the mutual substrate in the epidemiology of the pathogen.

Methodological aspects

An immuno-histological technique was developed to study spatio-temporal dynamics of internal mycelium of *Botrytis* spp. and *U. atrum* in both pathosystems. This technique features a unique differentiation between the mycelia of *Botrytis* spp. and *U. atrum* and has the potential for a much wider application in the field of fungal ecology. The availability of specific fungal labels seems as yet to be the most limiting factor but this situation is rapidly improving.

Each of the techniques used to quantify fungal biomass focusses on one specific aspect of fungal presence: antigen production, cell wall components, enzymatic activity, mycelial biomass or sporulation. Overall insight in the ecology of fungi can thus never be obtained by using one specific technique. It is recommended to study fungal ecology using different, appropriate, techniques. When possible different substrates should be included to account for substrate quality. Interactions with other fungi have to be included when the development of fungal communities is to be understood and/or manipulated. The labourious nature of this type of work urges however for a careful selection of the organisms and the tools to be used.

Head start experiments, derived from de Wit replacement series (1960), proved a valuable tool in assessing the nature and magnitude of the interaction between *B. cinerea* and *U. atrum*. In combination with a theoretical approach as demonstrated in Chapter 3, it provides qualitative as well as quantitative information on the system studied. Head start experiments can be used to screen potential new applications of *U. atrum* and provide information on the potential success of *U. atrum* as a biocontrol agent. Under controlled nutritional conditions this approach could be used to explore the effect of (manipulation of) the nutritional composition on the interaction between pathogen and *U. atrum*.

Conclusion

Results generated in this thesis have demonstrated that *U. atrum* is an effective biocontrol agent of *B. cinerea* in cyclamen with the potential to be effective in other crops and against other pathogens. Techniques to investigate the competitive interaction between the mycelia of *Botrytis* spp. and *U. atrum* have been developed and are applicable in other pathosystems and potentially in natural fungal communities. Application of these techniques in combination with a system analytical approach led to the identification of ecological key factors determining the biocontrol effect of *U. atrum*. Based on these key factors new pathosystems with a high potential for successful application of *U. atrum* against *Botrytis* spp. or other fungal pathogens can be selected. The key factors are:

1) The presence of a mutual niche (substrate) for pathogen and *U. atrum* in the target crop.
2) The competitive abilities of pathogen and *U. atrum* in their mutual niche.
3) The role of the mutual niche in the epidemiology of the pathogen.

For practical purposes, more information on the effect of changing environmental conditions, conidial densities and spatial distributions of *B. cinerea* and *U. atrum* conidia on the leaf surface is needed. For fundamental insight as well as practical application, the impact of nutritional factors on the ecology of the biocontrol system has to be investigated, key nutritional factors have to be identified and the quantitative relationship between mycelial colonization and sporulation has to be established.

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Summary

Plant pathogenic *Botrytis* spp. are the causal agents of economically important diseases in field and greenhouse vegetables, fruits, ornamentals, and other crops throughout the world. Control of *Botrytis* diseases is based on fungicide applications but remains difficult. Biological control of *Botrytis* spp. would be a valuable alternative or supplement to present control measures. The fungal saprophytic antagonist *Ulocladium atrum* is an effective biological control agent of *B. cinerea* in cyclamen under commercial conditions. The biocontrol effect of *U. atrum* is based on both species competitively interacting in plant tissue. The aim of the research presented in this thesis was to identify the ecological principles behind the interaction between *Botrytis* spp. and *U. atrum* and elucidate the mechanisms of competition. This knowledge is essential to understand the possibilities and limitations of the antagonist.

The general introduction (Chapter 1) presents the fungal pathogens *Botrytis elliptica* and *B. cinerea* and the antagonist *Ulocladium atrum* studied in this thesis. It summarizes problems with present control strategies of *Botrytis* spp. and introduces the theoretical background of biological control of fungal foliar pathogens. The state of the art of biological control of *Botrytis* spp. using *U. atrum* is treated in detail and supplemented with theoretical background on mycelial interactions.
Biological control of *Botrytis* spp. by *U. atrum* is effectuated through competitive interactions between both species in plant tissue. At tissue level, the niche in which these interactions take place was unidentified, the competitive abilities of both species in their mutual niche(s) were not quantified and the dynamic principles of the interaction were unknown. At plant and crop level, the qualitative relationship between mycelial interactions of *Botrytis* spp. and *U. atrum* at tissue level and disease etiology and epidemiology was not known.

At the start of the project a technique to quantitatively study mycelial interactions between *Botrytis* spp. and *U. atrum* in plant tissue was unavailable. In Chapter 2 the development of a technique to localize, differentiate and quantify the mycelia of *Botrytis* spp. and *U. atrum* is described. A combination of differential fluorescent labelling and image analysis was applied on cryostat sections of necrotic tissue. *Botrytis* spp. mycelium was labelled specifically using a genus specific monoclonal antibody and a secondary fluorescein conjugate. Wheat germ agglutinin conjugated to the fluorochrome TRITC was used to label the mycelium of *Botrytis* spp. and *U. atrum*. Image analysis was applied to measure the relative surface area of fluorescing hyphae of *Botrytis* spp. and *U. atrum* in the cryostat sections. A mathematical

conversion was derived and used to calculate the relative or absolute mycelial volume of both species in the section. Variance components studied in relation to sampling strategies provided insight in colonization patterns within and between leaves and allowed the design of efficient experimental designs for future experiments. Later, the technique was extended when a monoclonal antibody to *U. atrum* became available. The adapted technique is used in Chapter 3 and allows specific and thus direct quantification of the mycelia of both species. This technique can be adapted and applied to study fungal ecology in other systems. At present, its potential is mainly limited by the availability of specific labelling techniques for fungal mycelia.

Temporal dynamics of colonization of necrotic cyclamen leaves by *B. cinerea* and *U. atrum* and the mechanistic aspects of the interaction were studied at tissue level in Chapter 3. The technique described above was employed in combination with modified de Wit replacement series (head start experiments) and pairwise mycelial confrontations. Development of mycelial biomass and sporulation in time followed logistic patterns for both species. Maximum colonization and sporulation levels reached by the individual species were lower when both species were competing for the same substrate. Competition between the mycelia of *B. cinerea* and *U. atrum* reduced sporulation of both species but the reduction was smaller then expected based on equal inter- and intraspecific competition. Nutrient competition was identified as the dominant antagonistic mechanism. The spectrum of nutrients utilized by *U. atrum* was shown to include the whole spectrum of essential nutrients utilized by *B. cinerea*. Overall, the results clearly show that *B. cinerea* can be completely excluded from necrotic cyclamen leaf tissue as a result of pre-emptive resource capture by *U. atrum*.

At plant and crop level, the effect of pre-emptive or competitive colonization of mutual substrates by *Botrytis* spp. and *U. atrum* on disease development was unknown. In Chapter 4, uncolonized necrotic tissue, was identified as the only niche available to *B. elliptica* and *U. atrum* in lily crops and *B. cinerea* and *U. atrum* in cyclamen crops. The potential and actual effect of pre-emptive colonization of necrotic tissue by *U. atrum* in lily and cyclamen crops was investigated. The conidia of *B. cinerea* are only weakly pathogenic in cyclamen, and saprophytic colonization of necrotic tissue is an essential step towards mycelial infection of healthy plant parts. Pre-emptive colonization of necrotic tissue by *U. atrum* blocks this infection pathway and results in a reduction of disease equivalent to that achieved after

regular fungicide applications. The conidia of *B. elliptica* are highly pathogenic on lily and infect healthy lily tissue directly. The infection pathway utilizing necrotic tissue was shown to be insignificant to disease development in this pathosystem. Consequently, *U. atrum* applications aimed at blocking this infection pathway were ineffective against *B. elliptica* epidemics. Thus, the competitive capabilities of pathogen and antagonist in their mutual substrate and the role of this substrate in the epidemiology of the disease are ecological key factors determining the potential biocontrol effect of *U. atrum* applications.

CompFun, a spatially explicit dynamic simulation model describing the interaction between the mycelia of *B. cinerea* and *U. atrum* at leaf level is developed in Chapter 5. The model integrates basic information generated in the previous chapters and is used to study system behaviour and identify knowledge gaps. Spatial expansion by both fungi is well predicted by the model. The general system behaviour regarding temporal colonization was also captured by the model. Large variation in the experimental data was however not reproduced. The cause for this variation is unknown and should be identified in future work to better understand the system. Establishment of fungal colonies after conidial inoculation is not included in the model and also deserves future attention considering the likely sensitivity of this process to adverse environmental factors. A sensitivity analysis resulted in the practical recommendation to establish high and uniformly distributed antagonistic conidial densities on the leaves to maximise the biocontrol effect. Uniform and extensive coverage by *U. atrum* conidia is important because its spatial expansion abilities are substantially lower than those of *B. cinerea*. Minimal conidial densities and distributions with a satisfactorily biocontrol effect may be established in scenario studies.

A general discussion of the results is presented in Chapter 6. It is concluded that *U. atrum* is an effective biocontrol agent of *B. cinerea* in cyclamen and has the potential to be an effective biocontrol agent in other crops and against other necrotrophic pathogens. With the tools and knowledge developed in this thesis new pathosystems can be selected effectively to maximise the chance of success. Three criteria govern the potential success of *U. atrum* as a biocontrol agent in a new pathosystem:

1) Is there a niche (plant substrate) in which *U. atrum* competes with the pathogen?
2) Is the competitive ability of *U. atrum* sufficient to allow for competitive exclusion of the pathogen from this niche?
3) Is the niche from which the pathogen can be excluded essential for disease development?

If one of these questions is answered negatively, as was question 3 for the lily - *B elliptica* pathosystem, then the use of *U. atrum* holds little promise. Otherwise, *U. atrum* is promising.
Further, it is concluded that the cause of the experimentally observed variation in the temporal colonization patterns should be investigated as it is of fundamental importance to the understanding of the system. Environmental conditions were only marginally included in the present study. They are however likely have a significant impact on system behaviour and should be investigated to allow development of *U. atrum* as into an effective and reliable biocontrol agent.

Samenvatting

Plant pathogene *Botrytis* spp. veroorzaken wereldwijd veel schadelijke ziekten in o.a. vollegrondsgroenten, kasgewassen, fruit en siergewassen. Bestrijding van ziekten veroorzaakt door *Botrytis* spp. is gebaseerd op herhaalde bespuitingen met fungiciden maar blijft problematisch door het 'explosief' karakter van deze ziekten en het regelmatig optreden van fungicideresistentie. Biologische bestrijding vormt een waardevol alternatief of aanvulling op het huidige pakket bestrijdingsmaatregelen. De saprofytische, antagonistische schimmel *Ulocladium atrum* is een effectieve biologische bestrijder van *Botrytis cinerea* in commerciële cyclaam gewassen. Het bestrijdingseffect van *U. atrum* is gebaseerd op competitieve interacties tussen *Botrytis* spp. en *U. atrum* in plantenweefsel. Het doel van het onderzoek beschreven in dit proefschrift was het identificeren van de ecologische basis principes en het ophelderen van het mechanisme achter de competitie tussen pathogeen en antagonist. Deze kennis is essentieel om de mogelijkheden en beperkingen van de antagonist als bestrijder van *Botrytis* spp. te kunnen begrijpen.

In de algemene introductie (Hoofdstuk 1) worden de pathogenen *B. elliptica* en *B. cinerea* en de antagonist *Ulocladium atrum* die in dit proefschrift onderzocht worden, geïntroduceerd. Problemen met de huidige bestrijdingsmethoden van *Botrytis* spp. worden samengevat en de theoretische achtergrond voor biologische bestrijding van necrotrofe bladpathogenen wordt geïntroduceerd. De stand van zaken met betrekking tot biologische bestrijding van *Botrytis* spp. door *U. atrum* wordt besproken en aangevuld met theoretische achtergronden over interacties tussen mycelia. Biologische bestrijding van *Botrytis* spp. is gebaseerd op competitieve interacties tussen pathogeen en antagonist in plantenweefsel. De weefsels waarin deze interacties plaatsvinden waren nog niet geïdentificeerd en de competitieve verhoudingen tussen *Botrytis* spp. en *U. atrum* waren niet gekwantificeerd. Het competitie mechanisme was nog onvoldoende opgehelderd. Op plant en gewasniveau waren de kwalitatieve relaties tussen onderdrukking van kolonisatie op weefselniveau en het effect op de etiologie en epidemiologie van de resulterende ziekte onvoldoende bekend.

Bij de start van dit project waren geen methoden beschikbaar om interacties tussen mycelia van twee schimmels op weefselniveau te bestuderen. In Hoofdstuk 2 wordt de ontwikkeling van een methode beschreven waarmee mycelia van *Botrytis* spp. en *U. atrum* gelokaliseerd, onderscheiden en gekwantificeerd kunnen worden. Twee kleurtechnieken werden gecombineerd en toegepast op vriesmicrotoomcoupes van gekoloniseerd necrotisch weefsel. *Botrytis* spp. mycelium wordt specifiek gekleurd

met behulp van een genus-specifiek monoklonaal antilichaam gekoppeld aan een groen fluorescerende merker. Het totale mycelium wordt vervolgens gekleurd met behulp van een lectine gekoppeld aan het rode fluorochroom TRITC. Het lectine bindt specifiek aan chitine in de celwand van schimmels. Met behulp van beeldanalyse wordt vervolgens het relatieve oppervlak van de mycelia van *Botrytis* spp. en *U. atrum* in de coupe bepaald. Dit oppervlak wordt vervolgens omgerekend naar het relatieve of absolute volume van de afzonderlijke mycelia in de coupe m.b.v. een wiskundige transformatie. Bestudering van variantiecomponenten m.b.t. de bemonsteringsstrategie gaf inzicht in kolonisatieverschillen van het mycelium binnen bladeren en tussen bladeren. Met behulp van deze variantiecomponenten konden effectieve proefopzetten voor toekomstige experimenten ontworpen worden. Met het beschikbaar komen van een monoklonaal antilichaam voor *U. atrum* werd het in Hoofdstuk 3 mogelijk zowel *Botrytis* spp. als *U. atrum* specifiek te kleuren. Deze techniek kan breed toegepast worden in andere pathosystemen en niet pathogene interacties tussen schimmels. De belangrijkste belemmering voor bredere toepassing is de, snel verbeterende, beperkte beschikbaarheid van specifieke merkers voor schimmel mycelium.

De temporele dynamica van kolonisatie van necrotische cyclamenbladeren werd bestudeerd in Hoofdstuk 3, in samenhang met de mechanistische achtergrond van de interactie tussen *B. cinerea* en *U. atrum*. De in hoofdstuk 2 beschreven techniek werd toegepast in combinatie met gemodificeerde "vervangingsreeksen" en paarsgewijze confrontaties tussen de mycelia van beide soorten. Logistische patronen werden gevonden voor de ontwikkeling van intern mycelium en sporulatie voor beide schimmels. De maximum kolonisatie- en sporulatieniveaus waren lager indien beide soorten in competitie waren om hetzelfde substraat. Competitie tussen de mycelia van *B. cinerea* en *U. atrum* reduceerde de sporulatie van beide soorten, maar de reductie was kleiner dan verwacht op basis van gelijke inter- en intraspecifieke competitie. Competitie om nutriënten was het belangrijkste competitieve mechanisme. Het nutriëntenspectrum, beschikbaar in necrotisch cyclamenblad voor *U. atrum* omvatte, het hele spectrum essentiële nutriënten beschikbaar voor *B. cinerea*. Aangetoond is dat *B. cinerea* uitgesloten wordt van necrotisch weefsel als dit preventief door *U. atrum* is gekoloniseerd.

In Hoofdstuk 4 wordt ongekoloniseerd necrotisch weefsel in lelie en cyclaamgewassen geïdentificeerd als het enige substraat dat zowel geschikt is voor kolonisatie door *Botrytis* spp. als door *U. atrum*. Vervolgens werd het potentiële

effect en het werkelijke effect van preventieve kolonisatie van dit weefsel met *U. atrum* op de ontwikkeling van een *Botrytis* epidemie onderzocht. *B. cinerea* conidia in cyclamen bleken slechts zwak pathogeen. Saprofytische kolonisatie van necrotisch cyclamenweefsel door *B. cinerea* is een essentiële stap voordat het mycelium gezond weefsel kan infecteren. Preventieve kolonisatie van dit necrotisch weefsel door *U. atrum* blokkeert deze infectieroute en resulteerde in een onderdrukking van de ziekte gelijkwaardig aan de onderdrukking bereikt met behulp van fungiciden. *B. elliptica* conidia in lelie bleken sterk pathogeen en in staat gezond weefsel direct te infecteren. De infectieroute via necrotisch blad bleek van geen enkel belang voor de ontwikkeling van de ziekte. Het gevolg was dat toepassing van *U. atrum* geen effect had op de ontwikkeling van *B. elliptica* in lelie. Op plant en gewasniveau worden het bestaan van een gemeenschappelijk substraat en de rol van dit substraat in de cyclus van het pathogeen aangewezen als ecologische sleutelfactoren. Samen bepalen zij het potentiele effect van *U. atrum* toepassing op de ontwikkeling van ziekten veroorzaakt door *Botrytis* spp.

In Hoofdstuk 5 werd een model ontwikkeld, CompFun geheten, dat de kolonisatie van necrotisch cyclamenblad door de mycelia van *B. cinerea* en *U. atrum* beschrijft. Het model integreert de beschikbare kennis m.b.t de interactie tussen *B. cinerea* en *U. atrum* en wordt gebruikt om lacunes in de huidige kennis van het systeem op te sporen. Ruimtelijke uitbreiding van *B. cinerea* en *U. atrum* kolonies wordt door het model goed voorspeld. Het algemeen gedrag van het systeem m.b.t. temporele aspecten van substraatkolonisatie wordt eveneens goed gesimuleerd met het model. De grote variatie in experimentele data wordt niet door het model gereproduceerd. De oorzaken van deze variatie zijn onbekend. Verschillen in de kwaliteit van het substraat als voedingsbron voor *B. cinerea* en *U. atrum* zouden een belangrijke rol kunnen spelen. Identificatie van de oorzaak is essentieel voor een goed begrip van het systeem. Vestiging van kolonies, een proces volgend op kieming van conidia, was niet opgenomen in het model maar zou in toekomstig onderzoek meer aandacht moeten krijgen gezien de potentiele gevoeligheid van dit proces voor nadelige omgevingsinvloeden. Een gevoeligheidsanalyse van het model voor kleine veranderingen in de modelparameters resulteerde in de praktische aanbeveling om te streven naar hoge en zo uniform mogelijk verdeelde conidiëndichtheden van *U. atrum* op het blad omdat de ruimtelijke uitbreiding van *U. atrum* achter bleef bij die van *B. cinerea*. Minimale dichtheden en verdelingen die toch een goed bestrijdingseffect garanderen kunnen in toekomstige scenariostudies onderzocht worden.

Een algemene discussie van de resultaten wordt gepresenteerd in hoofdstuk 6. Geconcludeerd wordt dat *U. atrum* een effectieve biologische bestrijder van *B. cinerea* in cyclamen is en dat deze antagonist de potentie heeft om in andere gewassen en tegen andere pathogenen werkzaam te zijn. Met de technieken en systeemkennis beschreven in dit proefschrift is het mogelijk pathosystemen op een snelle en efficiënte wijze te screenen om zo de kans op succesvolle toepassingen te maximaliseren.
Drie voorwaarden zijn bepalend voor het potentiele succes *U. atrum* als biologische bestrijder:

1) Bestaat er een niche (substraat) in het gewas waarin pathogeen en antagonist concurreren?
2) Is *U. atrum* in staat om het pathogeen van dit substraat uit te sluiten?
3) Speelt het gezamenlijk substraat van pathogeen en antagonist een essentiële rol in de ontwikkeling van de ziekte.

Indien één van deze vragen met "nee" beantwoord wordt, zoals voor het pathosysteem *B. elliptica* - lelie, dan is het potentieel van *U. atrum* gering. Indien alle vragen met "ja" beantwoord worden biedt toepassing van *U. atrum* perspectief.

Voorts wordt geconcludeerd dat het opsporen van de oorzaken van de experimenteel gevonden variatie in de temporele kolonisatiepatronen van *B. cinerea* en *U. atrum* in necrotisch cyclamenblad essentieel is voor een beter begrip van het systeem voor zowel fundamentele als praktische toepassingen. Klimaatsfactoren werden in het huidige onderzoek slechts marginaal behandeld. Zij kunnen echter een grote potentiele invloed op het gedrag van het systeem hebben. Nader onderzoek naar de invloed van klimaatsfactoren op het bestrijdingseffect van *U. atrum* is daarom noodzakelijk om met deze antagonist een effectief en betrouwbaar biologisch bestrijdingsmiddel te kunnen ontwikkelen.

Curriculum vitae

Gerardus Johannes Theodorus Kessel werd geboren op 1 augustus 1964 te Oudenbosch. In 1983 behaalde hij zijn VWO diploma aan het Norbertus College in Roosendaal. Aansluitend begon hij een studie Plantenziektenkunde aan de Landbouwuniversiteit Wageningen. De doctoraalfase omvatte afstudeervakken bij de vakgroepen Fytopathologie, Landbouwplantenteelt en Nematologie. In 1987 deed hij in het kader van een stage 5 maanden onderzoek bij het Volcani Centre in Bet Dagan, Israël. Na het behalen van zijn doctoraaldiploma in 1989 werkte hij gedurende 3 maanden als projectmedewerker bij de vakgroep Fytopathologie waarna aansluitend de militaire dienstplicht werd vervuld. Vanaf 1991 tot en met 1994 werkte hij als wetenschappelijk onderzoeker bij het DLO-Instituut voor Plantenziektenkundig Onderzoek (IPO-DLO) aan alternatieve bestrijdingsmethoden voor de aardappelziekte veroorzaakt door *Phytophthora infestans*. Van 1995 tot en met 1999 werkte hij als onderzoeker in opleiding (OIO) bij het IPO-DLO en het Laboratorium voor Theoretische Produktie Ecologie van de Wageningen Universiteit aan het in dit proefschrift beschreven onderzoek.
Momenteel is hij werkzaam als onderzoeker bij het Instituut voor Plantenziektenkundig Onderzoek (IPO) en als toegevoegd onderzoeker bij het Laboratorium voor Theoretische Productie Ecologie.

List of publications

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