Epidemiology and Biological Control of Grey Mould in Annual Strawberry Crops

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PROPOSITIONS

Ulocladium atrum has the potential to control *Botrytis cinerea* on strawberries, provided that it is applied at the proper time and under suitable conditions.

This thesis

Male parts in strawberry flowers provide both the good and the bad.

This thesis

Detailed epidemiological studies are a prerequisite for effective use of biocontrol agents.

The forces toward true sustainability are guided by social concerns.

Most science is a desperate search for a supposed truth.

It is better to be pessimist than optimist. The pessimist is happy when he scores and when he does not score.

Millôr Fernandes

There is no virginity more unprotected than a blank sheet of paper.

Mário Quintana

... as a humanist, I agree with the internationalisation of the world, of the petroleum, of the capital of rich countries, of the nuclear arsenal of the USA, of the poor children, of the Louvre museum, and even of the Amazon. But, while the world treats me as a Brazilian, I will fight for the Amazon to be for us. Just for us.

The world for all by Cristovam Buarque

Aos que à vida me trouxeram

e

aos que, a poesia do viver comigo compartilham

ABSTRACT

Boff, P., 2001. Epidemiology and biological control of grey mould in annual strawberry crops. PhD Thesis, Wageningen University, Wageningen, the Netherlands.

Intensive crop production has led to various undesirable side effects. Strawberry production is typically very input-intensive, in particular with respect to fungicides. In this thesis we attempt to develop a control strategy for strawberry grey mould caused by *Botrytis cinerea* Pers. using an ecological approach. The epidemiology and biological control of grey mould using the antagonist *Ulocladium atrum* Preuss was studied during four years in an annual cropping system under field conditions. *U. atrum* sprayed throughout the season controlled grey mould on fruits effectively in five of eight experiments. On the phylloplane, *U. atrum* spore density declined exponentially with a relative rate of -0.10 day^{-1}. This means that sprays would need to be applied once a week for *U. atrum* to compete effectively with *B. cinerea* in dead leaf tissue. However, very little leaf debris was formed in annual strawberry and the pathogen sporulated on a maximum of 15.5 cm^2 of leaf area per plant. Crop sanitation by removing dead leaves did not affect the level of grey mould. These results demonstrate that crop debris was not a significant inoculum source for *B. cinerea* in this strawberry system. The presence of petals can facilitate the establishment of infection of *B. cinerea* on fruits, considering that 65-85% of them exhibited pathogen sporulation, and petal retention during fruit development was associated with 50% of the total grey mould. Therefore targeting the infection of *B. cinerea* on flower parts is more efficient for the control of grey mould than suppressing sporulation of the pathogen on crop debris. Spraying *U. atrum* during flowering was as effective as spraying from transplanting. A single application of *U. atrum* was effective in reducing grey mould when introduced at late flower- or early fruit stages. Multiple applications during flowering showed that twice weekly sprays gave better control than weekly sprays. The conidium concentration of *U. atrum* can be as low as 0.5×10^6 conidia ml^{-1} when applied at flowering.

Key words: Annual cropping system, biological control, *Botrytis cinerea*, epidemiology, grey mould, infection, petal, strawberry, *Ulocladium atrum*.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1. Agriculture in transition: ecological soundness of plant disease management

1.1. Current agricultural practices and the need for change

Over the last 50 years, agricultural practices with high material input have caused loss of topsoil, environmental contamination, and direct risks to farmer- and consumer health (Stinner and House, 1987; Reganold, 1988; Pinheiro et al, 1993). The amount of pesticides used in crop protection programmes has increased steadily, while effectiveness of such pesticides has decreased due to the development of resistance (Paschoal, 1979; Bull and Hathaward, 1986; Ghini, 1996). This is especially true for horticultural crops. Horticulture is by its nature more intensive than other crops since its products are more valuable per unit area, and consumers expect very high standards, particularly in appearance of the product. One of the horticultural crops grown most intensively is strawberry. For example, among 16 crops surveyed in Norway, strawberries received the largest number of pesticide applications (namely 10) per season (Saethre et al, 1999). As a result, pesticide residues are not uncommon in marketed strawberry fruit. For example, in an extensive survey of marketed strawberry fruit in Italy, about 1.5% had more than the allowable concentrations of pesticides (Branca and Loia, 2000). In total, 39 different pesticides were detected in the samples. Thus, it is understandable that the general public is concerned about pesticide residues and human health, although this fear is not justified in each and every country (van Bruggen, 1995). In general, concerns about food safety and environmental degradation have raised doubts about the sustainability of the intensive practices in contemporary agriculture (Tagliari, 1999; Hodges and Scofield, 1983).

Several alternatives are being developed, the most important being low-input or integrated farming systems (where input of synthetic fertilisers and pesticides are reduced) and organic farming systems (where synthetic inputs are avoided, while

organic matter conservation and biodiversity are enhanced). The first type of farming systems focuses on minimising mineral nutrient surpluses and pesticides released into the environment (Oomen et al, 1998). The last farming systems are ecologically based. They take into account the improvement of energy flow, the recovery of biodiversity, the production of healthy foods and the empowerment of farmers by using more internal resources according to their traditional and indigenous knowledge (Tagliari, 1999; Testa et al, 1996; Soule and Piper, 1992; Pimbert, 1999).

The development of an alternative to conventional agriculture, based on ecological principles, started all over the world soon after the first awareness of the negative impact of pesticides, but only recently did it become part of official programmes included in the public policies (Pinheiro et al, 1999; Niggli and Willer, 2000; Rowe, 1999; Fontana, 2000). In Santa Catarina State (BR) for example, soil erosion control was successfully implemented by micro-watershed planning, which involved some 38000 farms in the last 10 years. The project was based on local resources including soil management by using green manure, no tillage and cover crops. External inputs such as fertilisers decreased, whereas yields of several crops rose (Pretty, 1995). Some pesticides, however, are still used and can become a problem in water and soil contamination in the coming years (Tagliari, 1999).

Another example is strawberry production in California, which is one of the most intensive strawberry production areas in the world. The pending phase-out of methyl bromide as pre-plant fumigant has spurred a search for alternative pest control methods (Sances and Ingham, 1997). Although organic strawberry production has long been a small-scale operation next to the large-scale conventional strawberry production systems (Gliessman, et al, 1996), in recent years large strawberry producers have converted part of their operations to organic production. This has increased supplies of organic strawberries to such an extent that price differentials between organic and conventional fruit have been reduced substantially (Duniway, personal communication). This large-scale conversion may, however, contribute to an overall reduction in pesticide use.

Conversion of intensive agricultural systems to less intensive alternative systems often results in yield reductions. This is also the case for strawberry production (Gliessman et al, 1996). Yield reductions are frequently primarily due to

pests and diseases, especially during the transition period. In organic strawberry production, fruit diseases, in particular grey mould, and powdery mildew, were the main yield-limiting factors (Birkeland and Doeving, 2000). Since pests and diseases can still limit yields after conversion to organic farming, the development of alternative technologies to reduce the impact of these pests and diseases is crucial to improve sustainability (Paschoal, 1994; Verona et al, 1999).

1.2. Ecological plant disease management

Ecological plant disease management is generally based on the principle of prevention, which minimises the need for interventions during the cropping cycle (Stoll, 1987; Thurston, 1992). Improving soil health and fertility by raising the organic matter content, for example, may increase plant resistance and tolerance to pathogens (Chaboussou, 1980; Werner, 1999; Verona et al, 1999). Increasing biodiversity builds complexity in the food web and may suppress opportunistic species such as pathogens, which may otherwise develop into epidemics (Paschoal, 1994; Soule and Piper, 1992). Maintaining a broad genetic basis for cultivated plants allows the crop to adapt easily to different environmental conditions (Robinson, 1996). Using crop management practices such as spacing, inter-cropping and change in transplanting time will retard or reduce the rate of disease development, so that it does not reach the economic threshold (Zadoks and Schein, 1979; Thurston, 1992; Daugaard, 1999). Adding compost may suppress soilborne pathogens and enhance plant tolerance to airborne diseases as well (Hoitink and Boehm, 1999; Boff et al, 2000). Thus, healthy agro-ecosystems, in terms of stability and resilience in response to a disturbance or stress, should be the final aim in organic or ecological production systems (Van Bruggen and Semenov, 2000; Van Bruggen, 2000).

However, to maintain adequate yields during the transition period, when the agro-ecosystem itself is not yet able to suppress the development of pathogens, complementary practices during crop development may be needed (Tagliari, 1999). Moreover, even in long-term ecological farming systems, foliar plant diseases can remain problematic in humid climates (Van Bruggen, 1995). In that case, curative interventions will remain necessary to control these diseases. Such curative

interventions include the application of natural products, which are required to be in compliance with organic standard regulations (Schmidt and Haccius, 1998; Stopes et al, 2000). Products for plant disease control include silicates, extracts from compost, plants and propolis, homeopathic preparations, wood ash, bio-fertilisers, milk, etc (Boff et al, 1999; Bettiol, 1999; Tratch and Bettiol, 1997). In addition biocontrol agents can be applied.

Micro-organisms for biological control of plant diseases can be used in various ways, viz. conservation, augmentation and importation (Bellows, 1999). Conservation is a naturally operating phenomenon in ecological agro-ecosystems, because the need for intervention is minimised (Ehler, 1998). It can be enhanced by creating conditions that favour survival and reproduction of antagonists at the expense of pathogenic organisms (Windels, 1997; Bettiol et al, 1998). Augmentation, based on mass-cultivation of indigenous antagonists, can provide an effective tool for intervention at a particular critical time (Bellows, 1999). An example of this is the use of *Acremonium* spp. to control tar spot of coconut (*Coccostroma palmicola*) in Brazil (Sudo, 1989; Bettiol, 1996). Inundative application of biocontrol agents may have to be integrated with other measures, such as agronomic practices, to achieve satisfactory disease management by preventing pathogen establishment or controlling established pathogen populations (Maloney, 1995).

2. Strawberry crop and biological control of grey mould

2.1. Botany and cropping systems

The modern garden strawberry (*Fragaria x ananassa* Duch.) is a natural species hybridisation between the Scarlet (*F. verginiana* Duch.) and the Frutillar or Chilean strawberry (*F. chiloensis* (L.) Duch) (Galletta and Bringhurst, 1990). The morphology of the above-ground strawberry plant is summarised in Figure 1. Species of the genus *Fragaria* are described as perennial plant with leaves and flowers in a basal tuft originating from a so called crown.

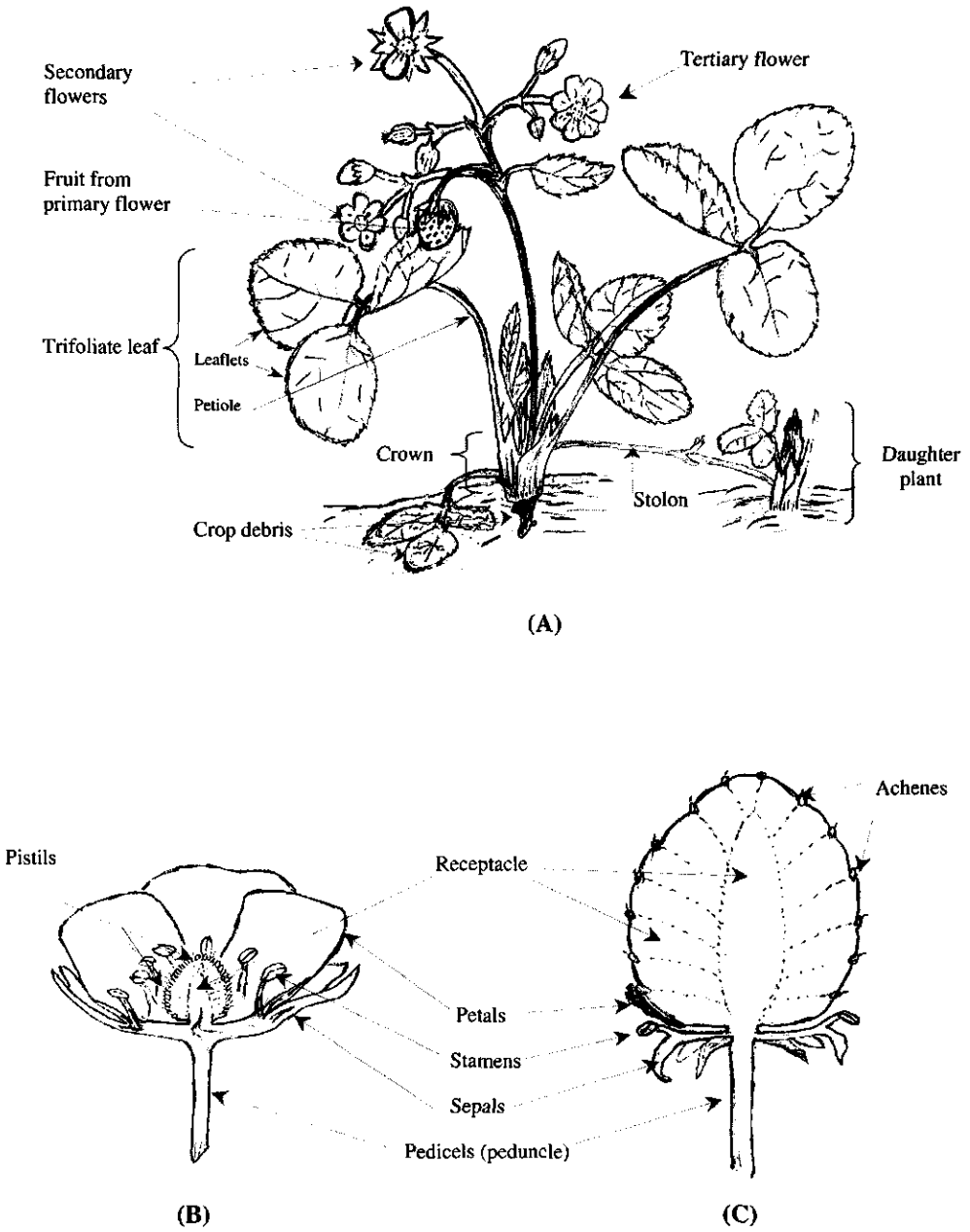

Figure 1. Morphology of strawberry plant with inflorescence and daugther plant (A), flower (B), and fruit (C). Modified after Galletta and Bringhurst (1990) and Strand (1994).

The crown of the strawberry plant is a shortened stem with closely spaced leaves. Between each leaf and the stem are auxiliary buds, which may develop into runners (stolons), branch crown, or flower branches (inflorescence) (Strand, 1994). Stolons are prostrated stems, which form daughter plants (transplants) at the node of them.

The inflorescence of strawberry is a determinate cluster of flowers that arises from a common peduncle by dichotomous branching immediately beneath the terminal flower. Consequently, flowers in the same inflorescence can be considered in a successive order of branching, namely primary, secondary, tertiary, and quaternary, which are close to the order of appearance on the principal branch (inflorescence) (Galletta and Bringhurst, 1990).

Despite the fact that species of the genus *Fragaria* are botanically described as perennial plants, strawberries in fruit production fields can be viewed as a perennial, biannual or annual crop by considering the time from transplanting till harvesting (Hancock, 1999; Rosati, 1991). Strawberry fruit production systems vary a lot around the world depending on germ plasm, environmental conditions, economic perspectives and farmer and consumer preferences (Rosati, 1991). Perennial cropping systems are preferred in some temperate regions such as Scandinavia and the United Kingdom, whereas annual crops can be found in the Mediterranean region and South America (Hancock, 1999). In Brazil, for example, transplants preferable produced in higher altitude regions (700 to 1000 m) during spring-summer (October to February), are transplanted from March to May. The crop continues development during autumn-winter and fruits are harvested from September onwards (Rebelo and Balardin, 1997). In the Netherlands, a particular system that uses waiting-bed transplants is adopted by most of the strawberry farmers. In such a system, the transplants are produced in beds from August to December and stored at -2 °C till transplanting the following spring or summer (Rosati, 1991). After transplanting, they may produce fruit in the same summer or be kept in the field to force a strong multi-crown (fruits are removed), which will enhance yields for the next spring. Although multi-crop planting (perennial) is less expensive to establish, annual cropping systems tend to predominate because both the exposure to pests and diseases and the maintenance costs are reduced, whereas the economic return per unit invested is higher (Galletta and Bringhurst, 1990; Rosati, 1991).

2.2. Pathogen and disease development

Grey mould, caused by *Botrytis cinerea* Pers. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel), is reported as an important disease of strawberry in any of the cropping systems used world-wide (Maas, 1984; Ghini and Vitti, 1993; Daugaard, 1999). *B. cinerea* is also a pathogen in several other crops that can result in disease with potential yield losses in grapes, ornamental plants, and nurseries of tree seedlings (Jarvis, 1980b; Pereira, 1989; Tatagiba et al, 1998). On strawberry crops, infection by *B. cinerea* may lead to blossom blight or grey mould on fruits. Grey mould on fruits, at harvest or post-harvest, is the major cause of yield losses in this crop. Inoculum sources of *B. cinerea* and the epidemiology of disease on strawberries according to the crop development is summarised in Figure 2.

B. cinerea has traditionally been considered as a non-specialised necrotrophic fungus that multiplies on debris of a broad range of plant species (Anderson, 1924; Jarvis, 1962a). There is, however, some physiological specialisation on different host plants (Likhachev et al, 1998). Flowering and fruit development are the suitable stages where *B. cinerea* starts infection leading to blossom blight or grey mould development in strawberry crops. Inoculum load of *B. cinerea* conidia during flowering and fruit development of strawberries is built up from screrotia, weed residues, and crop debris located inside or outside the strawberry fields (Fig. 2) (Jarvis, 1962a; Braun and Sutton, 1987). In perennial strawberry fields, necrotic tissue in the crop canopy was found to be the major inoculum source of *B. cinerea* (Braun and Sutton, 1987). However, this relationship was not yet reported for annual strawberry crops.

Strawberry infections leading to grey mould development on fruits can originate from latent infections of *B. cinerea* established during flowering or by direct infection during the development of fruits (Fig. 2) (Powelson, 1960; Pappas and Jordan, 1997).

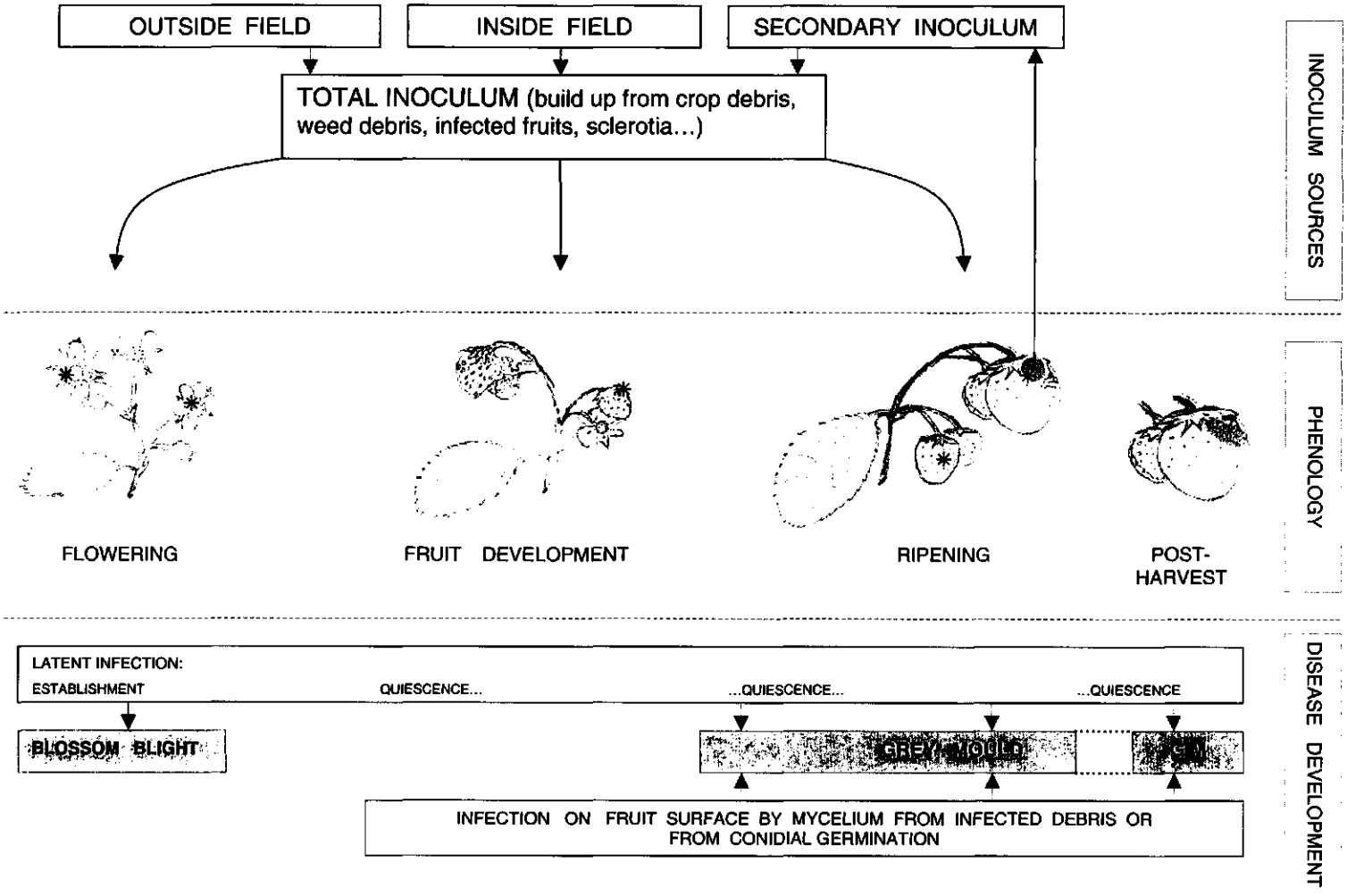

Figure 2. Inoculum sources and establishment of *Botrytis cinerea* (*) causing grey mould (GM) on strawberry fruits.

Infection of flower parts such as stamens and sepals may result in the establishment of a latent infection in the receptacle. After establishment, *B. cinerea* remains quiescent in young fruits (Jarvis and Borecka, 1968; Bristow et al, 1986). The quiescence of the fungus is attributed to the presence of proanthocyanidins, enzyme-inhibitory tannins, which are metabolised as maturation progresses (Jersch et al, 1989).

Colonised necrotic flower parts, such as petals adhering to the fruit surface or trapped between fruit and calyx, are potential sources of direct fruit infections (Wilkinson, 1954). Besides providing nutrients to the pathogen, flower parts that adhere to the fruit surface may allow water films to persist long enough for the post-germination infection process to be completed by the spores trapped on such material (Jarvis, 1962a). Conidia of *B. cinerea* freely landing on the fruit surface are of minor importance in grey mould development (Powelson, 1960; Sutton, 1995).

2.3. Biological control of grey mould

To manage grey mould by biological means, a considerable understanding of the cropping system, the disease epidemiology, the ecology and population dynamics of the antagonists, and of interactions among these variables, is required. The biocontrol system is highly dynamic, depending on the interaction between growth and development of the host, infection cycles and serial dispersal of the pathogen, quantitative shifts in populations of the biocontrol agent and indigenous organisms, and microclimatic fluctuations (Sutton, 1995).

Biological control of grey mould on strawberry has been approached by (1) slowing down the inoculum production of the pathogen or (2) interfering with the infection process (Bhatt and Vaughan, 1962; Tronsmo and Dennis, 1977; Peng and Sutton, 1991). The suppression of pathogen colonisation of necrotic tissues affecting the potential sporulation of pathogens can have the advantage of a longer interaction time between antagonist and pathogen as compared to interactions during the infection process (Fokkema, 1993). However, this has not been investigated in different cropping systems of strawberry.

Several micro-organisms have been tested for biological control of *B. cinerea* on strawberries. *Gliocladium roseum* was reported to control grey mould in perennial crops by suppression of the pathogen inoculum production and in greenhouse crops by spraying during flowering only (Sutton, 1995; Valdebenito-Sanhueza et al, 1997). *Aureobasidium pullulans* was found to compete for nutrients when applied during flowering, yielding effective control of strawberry rots (Lima et al, 1997). In other crops, *B. cinerea* has also been managed by using the antagonist *Trichoderma harzianum* (O'Neill et al, 1996), *Myrothecium verrucaria* (Zhang et al, 1994), and *Ulocladium atrum* (Köhl et al, 1995a) among others.

The fungal antagonist *U. atrum* has a high competitive ability to utilise necrotic tissue rapidly and extensively. Its mode of action in control of *B. cinerea* is predominantly by competition during colonisation of such necrotic tissue, which the pathogen uses as inoculum source or entry to healthy tissue (Kessel, 1999). However, the reliability of an antagonist in controlling foliage, flower and fruit diseases can be evaluated with confidence only in the cropping system or under closely representative conditions (Sutton, 1995). It was already demonstrated that *U. atrum* can significantly control *B. cinerea* in greenhouse crops of pot roses (Köhl and Gerlagh, 1999), cyclamen (Köhl et al, 2000) and geranium (Gerlagh et al, unpublished). *U. atrum* applications resulted also in significant reduction of grey mould under field conditions of grapevine crops (Schoene and Köhl, 1999). Effectiveness of a biological control agent depends on methods and strategies for introducing and maintaining the organism in the target crop (Sutton and Peng, 1993). Development of rational strategies for biocontrol treatments of the foliage for example, will require information on the population dynamics of introduced antagonists in relation to *B. cinerea* on the leaves and fruits. In an ideal biocontrol system, antagonists should be introduced only when and where they are needed, e.g. on the developing fruit, to minimise wasteful application of inoculum to non-targets (Sutton, 1995).

The scope of this thesis

The main purpose of the thesis was to develop a biocontrol strategy by which farmers could manage plant diseases in integrated and organic farming systems. The objectives of the research described in this thesis were to quantify (1) components of grey mould epidemics of annual strawberry crops using waiting-bed transplants and (2) the efficacy of the indigenous fungal antagonist *Ulocladium atrum* in controlling the disease under field conditions, either introduced all over the plants or targeting flowers only. Finally, the outcomes are also discussed with respect to potential use under Brazilian conditions, especially in Santa Catarina State.

In chapter 2, the relative importance of some inoculum sources of *B. cinerea* in epidemics of grey mould in annual strawberry crops is considered. Relationships between the inoculum production of *B. cinerea* on necrotic leaves, colonisation of flowers, and grey mould on fruits under field conditions are addressed.

In chapter 3, the efficacy of the fungal antagonist *U. atrum* in eight field experiments, at two locations during four years, is reported. The incidence levels of *B. cinerea* on flowers and grey mould development on fruits using *U. atrum*, fungicide, crop sanitation, or no intervention treatment are compared.

In chapter 4, the ecology of *U. atrum* in the strawberry phyllosphere is studied. In particular, the persistence, density and viability of conidia over time, and the competitive colonisation of the antagonist on leaves are reported.

In chapter 5, the role of petals in the establishment and development of grey mould on fruits is studied in detail. To this end, spatial distribution of *B. cinerea* sporulation on flower parts is assessed and symptom initiation of grey mould on fruits characterised. The effect of petal removal at different flower and fruit stages on development of grey mould is also investigated

In chapter 6, the efficacy of the antagonist *U. atrum* to control grey mould by a single application at different flower and fruit development stages is evaluated.

In chapter 7, grey mould epidemiology in annual cropping systems and the efficacy of the antagonist *U. atrum* are discussed. Special attention is devoted to the prospective use of the results of this research under conditions of Santa Catarina State, Brazil.

Chapter 2

EPIDEMIOLOGY OF GREY MOULD IN ANNUAL WAITING-BED PRODUCTION OF STRAWBERRY

Abstract

The epidemiology of *Botrytis cinerea* was studied in five annual strawberry crops using waiting-bed transplants, a system widely adopted in the Netherlands. On dead leaves of transplants the incidence of *B. cinerea* varied from 26.7 to 52.6%, but the area of potential sporulation was low (3.5 - 15.6%). During the crop cycle, the availability of necrotic leaf substrate for spore production of *B. cinerea* was generally low and varied between seasons and with the quality of transplants. *B. cinerea* sporulated on a maximum of 15.5 cm^2 of leaf area per plant, measured as potential sporulation. Conidium load of *B. cinerea* in the air in untreated crops did not differ from the air load outside the crop or in crops where all dead leaves had been removed. *B. cinerea* incidence on flowers ranged from 5 to 96%, but no correlation was found with the potential spore production on necrotic leaves. Grey mould at harvest varied from 1.4 to 11.3% and was correlated with the average precipitation during the harvesting period but not with *B. cinerea* incidence on flowers. Post-harvest grey mould ranged from 2.1 to 32.6% and was correlated with petal colonisation by *B. cinerea*. It can be concluded that in the studied annual cropping system with waiting-bed transplants, necrotic leaves were not a significant source of *B. cinerea* inoculum, unlike in other strawberry systems. Therefore, control measures of grey mould in this annual system should aim at protection of flowers and young developing fruits, rather than at reduction of inoculum production on leaf debris.

P. Boff, P. Kastelein, J. de Kraker, M. Gerlagh, and J. Köhl

INTRODUCTION

Strawberry (*Fragaria* x *ananassa* Duch.) can be cultivated in the open field as a perennial or annual crop. Annual crops are grown in spring, summer or autumn (Galletta and Bringhurst, 1990). A particular annual cropping system with waiting-bed transplants is used in North-western Europe, especially in the Netherlands. In this system, transplants are produced during autumn by planting fresh runners in August into waiting-beds. The transplants are harvested in December and subsequently stored at –2 °C (Rosati, 1991). Such cold-stored dormant transplants can be planted in the production field from April till July. Losses due to frost can be prevented in this cropping system by postponing the transplanting time. The risk of build-up soilborne diseases in the production field is also reduced because of the shorter cropping period. Moreover the choice of transplanting date allows the grower a flexible planning of crops according to labour load, risks of losses and expected prices.

Fruit rot caused by *Botrytis cinerea* Pers. (Teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) (Hennebert, 1973), known as grey mould, is an important world-wide disease in strawberry production systems. According to Powelson (1960), Jarvis (1962a), and Jarvis and Borecka (1968) grey mould is mostly due to infection from colonised senescent or dead flower parts by *B. cinerea*. After the development of fruit rot mycelium may invade adjacent strawberry fruits during ripening. Powell (1952) suggested early sprays of fungicides for eradicating over-wintering mycelium as efficient measure to protect blossoms and young fruits from conidial infections. In addition, Braun and Sutton (1987) demonstrated that the primary inoculum of *B. cinerea* leading to fruit rot in perennial strawberry crops is produced mainly on necrotic strawberry tissue inside the crop, present during flowering time. Several control measures of grey mould in perennial and over-wintering strawberry were proposed based on suppression of inoculum formation on strawberry plant debris, such as sanitation (Sutton et al, 1988), chemical control (Jordan and Pappas, 1977) and biological control (Sutton and Peng, 1993).

Quantitative epidemiology of *B. cinerea* was studied under controlled conditions (Bulger et al, 1987) and in the field (Jarvis, 1964; Wilcox and Seem, 1994). Significant correlation's were reported between weather variables during or

after the bloom period and fruit rot at harvest for perennial crops. Bulger et al (1987) found an increase of flower infections by *B. cinerea* with increasing wetness duration. The same authors could predict the incidence of grey mould as function of temperature and wetness duration during the flowering time. Sosa-Alvarez et al (1995) described the effect of temperature and leaf wetness on *B. cinerea* sporulation on dead strawberry leaves. In the annual cropping system using cold-stored waiting-bed transplants, the epidemiology of grey mould has not yet been studied. It may differ from over-wintering crops because the oldest leaves of the different transplants are mostly removed before planting, so that crops start with few necrotic leaves in spring. The dynamics of necrotic tissue after transplanting and its role as substrate for sporulation of *B. cinerea* may also differ from other cropping systems, because the transplants in the waiting-bed system are artificially kept in dormancy during 3-6 months at -2 °C.

The objective of our study was to investigate the relationships between the inoculum production of *B. cinerea* on necrotic leaves, colonisation of flowers by *B. cinerea*, and grey mould in the annual waiting-bed production system of strawberry under field conditions. Quantitative knowledge of the relative importance of the different inoculum sources will be helpful to identify the most appropriate targets for control strategies in such a cropping system.

MATERIALS AND METHODS

Field plots

All observations were made in the untreated control plots of five field experiments planted with strawberry cv. Elsanta, at a sandy soil near Wageningen, the Netherlands. The transplanting dates were (1) 10 April 1997; (2) 27 June 1997; (3) 06 May 1998; (4) 19 June 1998 and (5) 16 April 1999. The number of replicates was five and four for 1997 and 1998, respectively, and four to five treatments related to control strategies of grey mould were randomised within blocks. The plots in 1997 and 1998 consisted of 78 plants in an area of 4.5 m x 4.33 m, surrounded by a 10 m grass buffer to reduce inter-plot interference. In 1999, the experiment consisted of a single plot with the same plot size as in 1997 and 1998. Cold-stored dormant

transplants, kept at -2 °C until the day before planting, were transplanted in double rows with three plants per meter row length. Spacing of rows between double rows was 1 m and within double rows was 0.5 m. Sprinkler irrigation (1997) and a mist irrigation system (1998 and 1999) were used to ensure water supply for plant development in dry periods from transplanting till beginning of flowering. From beginning of flowering until the first harvest, strawberry plots were irrigated every second night (5 min per 90 min, in total 40 min per night) to stimulate epidemic development of *B. cinerea*.

Primary inoculum on transplants

Samples of senescent and dead leaflets and stolons were taken from the same bulk of cold-stored dormant transplants, used in experiment 3 (sample 1) and 4 (sample 2). Fifty units of each kind of leaflet and of each stolon, were washed thoroughly with tap water and put separately into plastic trays (50 cm length x 30 cm width x 7 cm height) with wet filter paper at the bottom and closed with a plastic bag. The incidence and the area with *B. cinerea* sporulation was estimated for each leaflet or stolon unit, using a stereomicroscope at 10 x magnification, after 14 days incubation at 18 °C in the dark.

Strawberry phenology

The phenology of strawberry crops was determined by periodic non-destructive measurements from transplanting until harvest, on four (1997) and five (1998) plants, arbitrarily selected and labelled per plot. In the single plot in 1999, ten plants were monitored. The numbers of flowers and fruits per plant were counted on dates shown in Figure 1. The area of necrotic leaf tissue was estimated by measuring the length and width of each necrotic leaf part with a millimetre scale ruler and calculating the area as a rectangle. The green leaf area per trifoliate leaf was estimated by a calibrated function, measuring length (L) and width (W) of the central leaflet (TLA= 268 + 1.903 L * W; R^2 = 0.94) c.f. Jensen et al (1977). Averages of green and necrotic leaf areas and number of flowers per plant per replicate were used for statistical analysis (Fig. 1).

Assessment of *B. cinerea*

Colonisation of necrotic leaves

Necrotic leaflets were incubated under moist conditions to assess the colonisation of the substrate by *B. cinerea* and the potential sporulation of the pathogen. Six samples were collected during experiment 1, on 13 May, 22 May, 30 May, 6 June, 20 June and 9 July, 1997; four samples in experiment 2, on 22 July, 29 July, 5 August and 14 August, 1997; three in experiment 3, on 7 May, 8 June and 16 June, 1998; three in experiment 4, on 20 June, 21 July and 4 August, 1998. No sporulation assessment on necrotic leaves was done in experiment 5. For each sample, 10 to 40 half or whole necrotic leaflets were harvested per replicate, so that not more than one leaflet per plant and not more than 5% of the total necrotic leaf area was removed per plot. This limit was considered to be the maximum that could be removed without significantly disturbing the substrate availability of *B. cinerea* in the field. The harvested leaflets per sample were put into a plastic tray (50 cm length x 30 cm width x 7 cm height) with wet filter paper at the bottom and closed with a plastic bag. The area with sporulation of *B. cinerea* was estimated for each leaflet, using a stereomicroscope, at 10 x magnification, after incubation for 14 days at 18 °C in the dark. The area colonised by *B. cinerea* per plant was estimated from the total necrotic area per plant and the proportion of sporulating area at the time of assessment. Interpolation was done for the purpose of correlating with other variables when assessment dates differed.

Flower colonisation

Samples of 20 to 30 flowers per plot were arbitrarily collected each from a different plant on two (exp. 1-2 and 5) or three (exp. 3-4) sampling dates (Table 2). Only flowers with brown anthers and with petals still attached were sampled. The flowers were put into plastic boxes (22 cm length x 14 cm width x 5 cm height) with wet filter paper at the bottom and were incubated at 18 °C for 14 days in the dark. All flower parts, except peduncles, were examined for the presence of sporulation of *B. cinerea* with a stereomicroscope, at 10-100 x magnification. Flowers were considered colonised by *B. cinerea* when at least one conidiophore with conidia was present on any of the flower parts. The incidence of *B. cinerea* sporulation on flowers was

calculated per sample per plot. In addition to the assessment of the incidence of *B. cinerea* on flowers, the area colonised by *B. cinerea* was estimated on petals for separate samples in experiments 3, 4 and 5. Twenty to 25 senescent petals were arbitrarily collected per sample per plot, each petal from a different plant. The number of sampling dates was three (11 June, 20 June and 2 July 1998), four (21 July, 2 August, 5 August and 11 August 1998) and five (24 May, 27 May, 28 May, 31 May and 4 June 1999) for experiments 3, 4 and 5, respectively. No separate sampling of petals was done in experiments 1 and 2. The petals were placed on water agar (1.5% of agar) in sterile plastic petri dishes (Ø14 cm) and were incubated for 14 days at 18 °C in the dark. The percentage area with *B. cinerea* sporulation was estimated for each petal using a stereomicroscope, at 20 x magnification.

Fruit rot

Grey mould was determined at harvest by counting the number of rotten fruits with *B. cinerea* symptoms (field fruit rot) and the total number of healthy fruits harvested per plot at each harvesting date. All ripe healthy fruits and all fruits with *B. cinerea* symptoms were picked twice per week. Healthy fruits were always picked separately from diseased fruits to avoid contamination during harvesting in view of post-harvest evaluations. Diseased fruits with non-specific symptoms were put into moist chambers for 72 hours at 18 °C in the dark, to allow development of specific symptoms. Post-harvest fruit rot was assessed using a sub-sample of ripe symptomless fruits, consisting of twenty arbitrarily selected fruits per plot. Fruits of each replicate were put into a plastic tray (50 cm length x 30 cm width x 7 cm height) with wet filter paper on the bottom. Each tray was closed within a plastic bag and incubated at 4 °C during five days in the dark and subsequently for more three days at 18 °C. After incubation rotten fruits with *B. cinerea* sporulation on any part of the fruit were counted.

Spore load in the air

The spore load with conidia of *B. cinerea* in the air was monitored using Rotorod samplers Mod. 20 (Sampling Technologies, Minnetonka, USA) with a non-retracting collector. Rotorods were positioned at 0.3 m height in the centre of an untreated plot

and 25, 50 and 50 m outside the strawberry plot in the grass buffer for the years 1997, 1998 and 1999, respectively. Runs were carried out on two, five, three and six days in experiments 2, 3, 4 and 5, respectively. Two to six runs per day with 15 min duration per run were carried out between 9:00 and 15:30 hours, which is the most likely period for spore release of *B. cinerea* (Jarvis, 1980a). In experiments 3 and 4, the spore load was also assessed in a plot in which all senescent and necrotic leaflets had been removed twice per week from transplanting till first harvest. The spores of one rod per run were stained with cotton blue (2 ml lactic acid + 4 ml glycerol + 1.5 ml cotton blue at 1% + 2 ml demi water) and conidia of *B. cinerea* counted on the 22 mm upper part of a rod using a microscope, at 200 x magnification. The number of conidia of *B. cinerea* per cubic meter of air was calculated per run and as an average per sampling day (Sampling Technologies, 1989).

Microclimatic conditions

Relative humidity and temperature were monitored with an electronic sensor (Pow 8-35 VDG, Rotronic AG, Bassersdorf, Switzerland) positioned at 30 cm height within a strawberry plot. Precipitation was measured by a rain gauge (Casella, London, UK). Wetness of necrotic leaves was measured in the canopy at 5 cm height, with leaf wetness sensors developed by Köhl et al (1995a). Data were stored each half-hour by a data-logger (Delta-T Devices Ltd, Camb., UK).

Data analysis

Statistical analysis was performed with Genstat 5 version 4.1 (Numerical Algorithm Group, Inc., Oxford, UK). Correlation analysis among epidemiological and weather variables was done by calculating the Pearson (r) and Spearman (r_s) correlation coefficients (Snedecor and Cochran, 1989). Quantitative correlation analysis was done between predicted values of *B. cinerea* sporulation using models developed by Sosa-Alvarez et al (1995) and estimates of the area colonised by *B. cinerea* on necrotic leaves, and flower incidence. Equation 7 (R^2=0.90) from Sosa-Alvarez et al (1995) was applied to our data, to predict sporulation of *B. cinerea* on strawberry leaf residue as a function of observed wetness duration and temperature. Quantitative

correlation analysis was also done between measured grey mould and predicted values obtained by models described by Bulger et al (1987). Equation 5 (R^2=0.79) from Bulger et al (1987) was applied to our data to predict the proportion of ripe strawberry fruit infected by *B. cinerea* as a function of observed temperature and wetness duration during flowering time.

RESULTS

Substrate availability and inoculum production of *B. cinerea*

On cold-stored transplants the incidence of *B. cinerea* was 26.7% (sample 1) and 52.6% (sample 2) on dead leaves and 29% (sample 1) and 32.2% (sample 2) on stolons. The incidence of *B. cinerea* on senescent leaves was 30.3% (sample 1) and 75% (sample 2). The potential sporulation area of *B. cinerea* on dead leaves was 3.5% and 15.6% for sample 1 and 2, respectively.

The dynamics of necrotic strawberry leaf tissue showed similarity among experiments 1, 2 and 5 and between experiments 3 and 4 (Fig. 1). In experiments 1, 2 (1997) and 5 (1999) the amount of necrotic leaf tissue increased steadily after transplanting but was below 25 cm^2 per plant until beginning of flowering and increased further during flowering. This trend was not observed in experiments 3 and 4 (1998) where more necrotic leaf tissue was found during the first weeks after transplanting whereas the presence of necrotic tissue was lower at flowering.

The area of necrotic leaves with potential sporulation of *B. cinerea* followed the same dynamics as the total area of necrotic leaf tissue in experiments 2 and 3 but not in experiments 1 and 4. The maximum area of potential sporulation of *B. cinerea* per plant was 15.4 cm^2 found in experiment 3, 14 days after transplanting. In experiment 4, the maximum potential sporulation area of *B. cinerea* was at transplanting time and decreased till the end. In experiment 1, the peak of *B. cinerea* sporulation potential was observed during early flowering but then decreased while the amount of dead tissue increased.

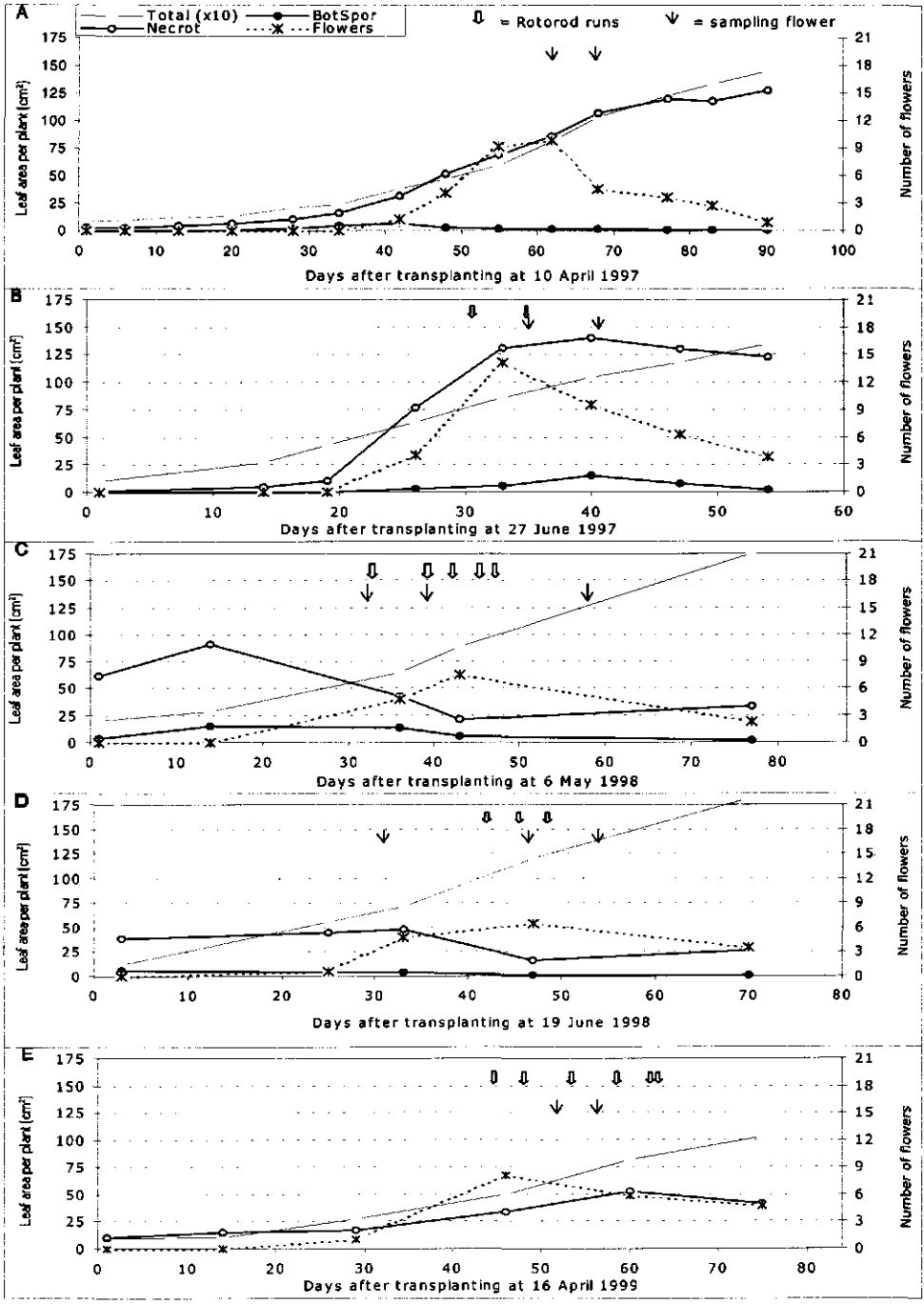

Figure. 1. Total leaf area x 10 (Total), total necrotic leaf area (Necrot), and area of necrotic leaf tissue with potential sporulation of B. cinerea (BotSpor) per plant (Y1), and number of flowers (Y2) per strawberry plant. Experiment 1, April-July, 1997 (A), experiment 2, June-August, 1997 (B), experiment 3, May-July 1998 (C), experiment 4, June-September, 1998 (D), experiment 5, Apr-July, 1999 (E). Runs of Rotorods and sampling of flowers are indicated.

In relation to the phenology of flowering, the potential sporulation of *B. cinerea* reached its maximum when the first flower appeared in experiments 1 and 3, at middle flowering in experiment 2, and at transplanting time in experiment 4.

Conidium load in the air during flowering

The average number of conidia per m^3 of air was generally very low (Table 1). Only in one of 43 runs carried out in the different experiments, the concentration was higher than 100 conidia per m^3 of air. A higher conidium load of *B. cinerea* inside the crop as compared to outside the crop was found on 10 of 16 days, given a proportion of 0.63 on which runs were carried out. Considering the large 95% confidence interval of the proportion (0.35,0.85) these differences were not significant. Furthermore, differences in spore load between inside and outside the crop were small, generally below 10 conidia m^{-3}. In the additional plots of experiments 3 and 4 in which senescent and dead leaves were regularly removed, the conidium load was not lower than in untreated plots (Table 1).

Table 1. Air load of conidia of *Botrytis cinerea* sampled by Rotorods during flowering in annual strawberry crops. Proportion of days with more conidia trapped inside crops than outside or in a cleaned plot, without dead strawberry debris. Wageningen, the Netherlands.

Experiment		Number of spores per m^3 of air per run		Proportion of days with Inside crop > Outside crop	Proportion of days with Inside crop > Cleaned crop
		Inside crop	Outside crop[1]		
Exp. 1	1997	-	-	-	-
Exp. 2	1997	19.6	11.2	0.50	-
Exp. 3	1998	8.2	5.8	0.60	0.40
Exp. 4	1998	21.7	4.5	0.67	0.33
Exp. 5	1999	16.3	10.2	0.67	-
Pooled data				0.63[2]	

[1] Rotorods were located at 25 m (1997) or 50 m (1998, 1999) from strawberry crop.
[2] Confidence interval is 0.35 – 0.85 (95%).

Flower colonisation

The incidence of *B. cinerea* on flowers varied in the five experiments, from 5.1% (exp. 1, sample 1, 1997) to 96% (exp. 2, sample 2, 1998) (Table 2). The incidence of *B. cinerea* on flowers increased in time in all experiments except in experiment 4.

Table 2. Incidence of flower colonisation by *Botrytis cinerea*, total necrotic leaf area, potential sporulation area on leaves at date of flower sampling and cumulative precipitation during 5 days before sampling of flowers. Wageningen, the Netherlands.

Experiment / sample date	*Botrytis* incidence in flowers (%)	Necrotic leaf area per plant (cm^2)		Precipitation, -5 to 0 days before sampling of flowers (mm)
		Total	with potential sporulation of *B. cinerea*	
Exp. 1 / sample 1 11-Jun-1997	5.1	86.3	1.9	89.4
Exp. 1 / sample 2 17-Jun-1997	14.9	108.0	1.7	91.8
Exp. 2 / sample 1 29-Jul-1997	76.2	123.6	4.1	11.0
Exp. 2 / sample 2 4-Aug-1997	96.0	137.9	14.5	7.8
Exp. 3 / sample 1 8-Jun-1998	63.7	49.2	16.5	127.6
Exp. 3 / sample 2 15-Jun-1998	70.0	30.1	8.7	40.6
Exp. 3 / sample 3 2-Jul-1998	81.3	26.1	4.9	91.8
Exp. 4 / sample 1 21-Jul-1998	83.7	48.0	3.7	4.4
Exp. 4 / sample 2 5-Aug-1998	75.0	16.5	0.8	46.6
Exp. 4 / sample 3 11-Aug-1998	60.1	19.2	0.7	109.0
Exp. 5 / sample 1 6-Jun-1999	60.2	88.0	-	35.6
Exp. 5 / sample 2 9-Jun-1999	65.2	97.1	-	24.6

Total necrotic leaf area per plant, at the time of flower sampling, varied from 16.5 to 137.9 cm^2. The leaf area per plant colonised by *B. cinerea* at the time of flowering was below 10 cm^2 per plant, except in experiment 2, sample 2 and in experiment 3, sample 1 (Table 2). No correlation (n=12) was found between total necrotic leaf area or leaf area colonised by *B. cinerea* and the incidence of *B. cinerea* on flowers. The daily average precipitation (rain + irrigation) in the five days before sampling of flowers showed high variation between 4.4 and 127.6 mm. No correlation (n=12) was found between the incidence of *B. cinerea* on flowers and climatic variables such as daily precipitation and leaf wetness duration before sampling of flowers and neither between incidence of *B. cinerea* on flowers and area of potential sporulation of *B. cinerea* at sampling of flowers (Table 2). The predicted levels of *B. cinerea* sporulation on necrotic leaves based on Sosa-Alvarez's model (equation 7; Sosa-Alvarez et al, 1995) and the leaf area colonised by *B. cinerea* did not positively correlate as expected with *B. cinerea* incidence on flowers assessed in these experiments ($r = -0.71$, $n=7$). Potential sporulation area of *B. cinerea* on petals was higher in 1998 than in 1999 (Table 3).

Grey mould

Fruit rot at harvest varied from 1.4% (exp. 2) up to 11.3% (exp. 4) (Table 3), whereas post-harvest grey mould showed values between 2.1 and 32.6%. Post-harvest fruit rot was mostly higher than fruit rot at harvest time and no correlation was found between harvest and post-harvest fruit rot. Fruit rot at harvest was significantly correlated with daily average precipitation during the harvest period ($r_s = 0.9$; $P<0.05$), but post-harvest fruit rot was not. The incidence of *B. cinerea* on flowers was better correlated with grey mould at post-harvest ($r=0.35$, $n=5$, $P>0.05$) than at harvest time ($r=-0.35$, $n=5$, $P>0.05$) but neither of the correlation was significant. The area of *B. cinerea* sporulation on petals assessed in three experiments, was significantly correlated with post-harvest fruit rot ($r_s=1.0$; $P<0.05$) but not with fruit rot at harvest. A significant correlation between the predicted values of fruit rot according to Bulger's model (equation 5) (Bulger et al, 1987) and the percentage of fruit rot observed in the field was only found in exp. 3 ($r_s=0.71$; $P<0.05\%$), but not for post-harvest fruit rot.

Table 3. Grey mould at harvest (HarvRot), grey mould at post-harvest (PostRot), potential sporulation of *B. cinerea* on necrotic leaves (LPS), *Botrytis* incidence on flowers (FBI), potential sporulation area for *B. cinerea* on petals (Petal), average daily precipitation during flowering and average daily precipitation during harvesting observed during 5 experiments. Wageningen, the Netherlands.

Experiment	HarvRot (%)*	PostRot (%)*	LPS (%)*	FBI (%)*	Petal (%)*	Average precipitation (mm day^{-1}) during	
						flowering	harvest
Exp. 1	9.5	13.1	9.4	9.9	-	9.6	5.5
Exp. 2	1.4	26.9	5.6	86.1	-	2.0	3.0
Exp. 3	10.1	10.4	22.4	71.7	20.3	14.6	6.2
Exp. 4	11.3	32.6	9.5	72.9	29.4	9.9	11.3
Exp. 5	11.2	2.1	-	62.7	5.8	12.5	5.6

* Averages for several sample times per experiment.

DISCUSSION

Inoculum production on necrotic leaves inside the crop

Grey mould on strawberry fruits has been reported as closely related to flower colonisation by *B. cinerea* and subsequent establishment of latent infections (Jarvis, 1962a), rather than to pathogen penetration of healthy fruit surfaces (Powelson, 1960). According to Jordan (1978) and Braun and Sutton (1987), the inoculum pressure of *B. cinerea* during flowering largely depends on conidia produced on dead strawberry tissue inside the perennial strawberry crop. In our study, the area of necrotic strawberry leaves increased rapidly from beginning of flowering till after the peak of flowering in three experiments (experiments 1, 2 and 5) or from transplanting

until beginning of flowering (experiments 3 and 4). These two different patterns are likely to be caused by differences in the quality of the transplants and the growing conditions after transplanting. For instance, planting of weak transplants resulted in a relatively large amount of necrotic leaf area soon after transplanting (experiments 3 and 4), but these leaves decomposed rather quickly and had almost disappeared at flowering time.

The senescing and dying leaves in our experiments were mostly those produced during autumn of the previous year, i.e. during the waiting-bed transplants production period. This generation of leaves is similar to that reported by Braun and Sutton (1988) as the major source of conidial production of *B. cinerea* during flowering. The incidence of *B. cinerea* was also high on cold-stored transplants. Nevertheless, only in experiment 2 the potential sporulation area of *B. cinerea* developed similarly to the total necrotic leaf area. Sutton et al (1988) also did not find a relationship between the amount of dead leaf tissue present in the crop and the potential sporulation of *B. cinerea* on such tissue. Moreover, sporulation on necrotic leaves in the field was hardly observed in our five experiments with waiting-bed transplants (data not presented), despite the conducive abiotic conditions created by regular irrigation (Sosa-Alvarez et al, 1995). More variables may affect the spore production of the fungus besides temperature and wetness duration, for instance the quality of leaf substrate. In the field, we observed sporulation of *B. cinerea* more frequently on necrotic tissue of young leaves than on fully expanded necrotic leaves (data not presented).

The amount of necrotic leaf residue produced in the annual strawberry crops in our experiments appears to be much less than in perennial strawberry production systems. The maximum we found was a necrotic leaf area of c. 150 cm^2 per plant, which is equivalent to a dry weight of c. 2 g per plant. For perennial crops, Sutton et al (1988) reported dry weights of dead foliage of 10-25 g per plant depending on crop management practices, while Jarvis (1962a) found that individual strawberry plants often harboured 200-300 g of debris. In summary, given the relatively low amount of necrotic leaf substrate for spore production and the low levels of actual and potential spore production observed on these leaves, necrotic leaves were most probably not an important inoculum source of grey mould in our experiments. This is supported by

the absence of significant differences in air load of conidia inside and outside the crops, and between crops with and without necrotic leaves (Table 1). In this respect the epidemiology of *B. cinerea* in the annual waiting-bed strawberry production system differs markedly from the epidemiology as described for perennial and over-wintering strawberry crops.

Colonisation of flowers by *B. cinerea*

Incidence of *B. cinerea* on flowers was high in almost all experiments. Ten of 12 samples had an incidence of *B. cinerea* more than 60%. According to Braun and Sutton (1987, 1988) the inoculum for flower colonisation by *B. cinerea* in spring originates mainly from necrotic leaves inside the crop. We did not find significant correlations between the incidence of *B. cinerea* on flowers and the total necrotic leaf area present during flowering, the leaf area with potential sporulation of *B. cinerea*, or the predicted amount of sporulation based on colonised leaf area and weather conditions (Sosa-Alvarez et al, 1995). This is not surprising, given our previous conclusion that spore production on necrotic leaves in our experiments was insignificant, even though considerable amounts of *B. cinerea* were found on transplants before planting. Despite the low amount of inoculum produced on necrotic leaves, the overall air load of *B. cinerea* conidia was sufficient to result in an average incidence of *B. cinerea* on 60% of the flowers. This confirms the statement of Berrie et al (1998) that the availability of inoculum of *B. cinerea* is unlikely to be a limiting factor determining disease incidence in strawberry.

Grey Mould

In none of our experiments a correlation between grey mould at harvest and the potential production of conidia inside the crop or incidence of *B. cinerea* on flowers could be demonstrated. Post-harvest fruit rot however, was significantly correlated with the level of petal colonisation by *B. cinerea*. This is probably due to latent infections of young fruits caused by infected petals (Powelson 1960, Jarvis 1962a, Bulger et al, 1987), which require additional favourable conditions to express symptoms on fruits. In our experiments, the daily average of precipitation during harvesting time was significantly correlated with fruit rot at harvest. Apparently, the

occurrence of conducive conditions in the field before harvest explains the variation in pre-harvest grey mould between cropping seasons better than the level of inoculum present. Jarvis (1964) found also a high correlation between fruit rot and pre-harvest rainfall. The same author suggested that the transition of latent mycelium in the fruit from a quiescent to an aggressive phase is hastened by an increased water content which can be facilitated by rain. Similarly, Wilcox and Seem (1994) found that the rainfall during the green fruit stage plus pre-harvest period was one of the most important variables for predicting grey mould incidence at harvest.

When we applied Bulger's models (Bulger et al, 1987) to predict pre- and post-harvest fruit rot as a function of leaf wetness and temperature during flowering, we obtained a significant correlation between predicted and observed levels in only one case (pre-harvest fruit rot in experiment 3). These poor results can be partly attributed to the absence of conducive conditions for symptom expression in the case of pre-harvest fruit rot. An additional explanation, in particular for the lack of correlation with observed levels of post-harvest fruit rot, is that the predictions by Bulger's models assume a constant and sufficient presence of inoculum, whereas in our experiments the inoculum load fluctuated daily. Moreover, in the experiments of Bulger et al (1987) grey mould was assessed on fruits originating from flowers that were inoculated and incubated in one particular stage of development, while in our experiments several generations of flowers (5-8 days) resulted in a single harvest. Thus, during the periods conducive to infection in the field, the flowers were in different stages of development and differed in susceptibility (cf. Jarvis and Borecka, 1968). In conclusion, we found that in the waiting-bed cropping system inoculum production of *B. cinerea* on plant debris inside the crop was insignificant. The level of grey mould on strawberry fruits in the field depends more on conducive conditions for disease during fruit development and ripening than on the incidence of *B. cinerea* on flowers. However, when conditions for symptom expression are suitable, the level of grey mould is determined by the level of flower colonisation by *B. cinerea*, as was demonstrated by the correlation between petal colonisation and post-harvest grey mould. Control strategies of grey mould, such as biological control, in annual strawberry cropping systems using waiting-bed transplants should aim to protect the flowers from colonisation by inoculum of *B. cinerea*.

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Chapter 3

BIOLOGICAL CONTROL OF GREY MOULD WITH *ULOCLADIUM ATRUM* IN ANNUAL STRAWBERRY CROPS

Abstract

The efficacy of the fungal antagonist *Ulocladium atrum* to control grey mould in annual strawberry crops under field conditions was investigated. Eight field experiments were conducted with strawberry cv. Elsanta, during the summer seasons of 1996-99, in Breda and Wageningen, the Netherlands. Treatments included control, fungicide programmes, *U. atrum* spray programmes and crop sanitation. *U. atrum* spray programmes effectively reduced grey mould at harvest in five of eight experiments. Sprays of *U. atrum* starting at transplanting resulted in better control of grey mould than sprays starting at the beginning of flowering only in one of five experiments. Crop sanitation did not affect the level of grey mould, which demonstrated that strawberry leaves were not a significant inoculum source for *B. cinerea* in this annual cropping system. Twice weekly sprays gave better control than weekly sprays of *U. atrum* irrespective of the suspension concentration. The conidial concentration of *U. atrum* during flowering applications can be reduced up to 0.5 x 10^6 conidia per ml without losing efficacy of antagonist. These results suggest that *U. atrum* can be effective in reducing grey mould in strawberry crops, and further studies on the use of the antagonist in annual systems should consider flowering time as the best period of spraying this antagonist.

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INTRODUCTION

Grey mould of strawberry (*Fragaria x ananassa* Duch.) incited by *Botrytis cinerea* Pers. can cause severe yield losses in any of the strawberry cropping systems used world-wide (Maas, 1984). The non-specialised necrotrophic *B. cinerea* can multiply on debris of a broad range of plant species (Anderson, 1924; Jarvis, 1962a) and disseminate as conidia. Senescent flower parts may facilitate the infection of *B. cinerea* on developing fruits leading to fruit rot (Jarvis, 1962a).

In the Netherlands, the majority of strawberry field crops are grown using the so-called waiting-bed transplant system (Rosati, 1991). This cropping system is also adopted in other North-west European countries. In such a system, the transplants are produced in waiting-beds from August until December and stored at -2 °C till transplanting in the following spring or summer (Rosati, 1991). Advantages of this system are: (i) no plant losses during winter; (ii) flexible planting schedule resulting in harvest periods with higher expected product prices; (iii) lower risk of build-up of populations of soilborne pathogens because of the reduced cropping period and; (iv) better stand quality by selecting transplants that developed a strong multi-crown during the waiting-bed time.

Necrotic tissue in the strawberry canopy has been found to be the major inoculum source of *B. cinerea* in perennial strawberry fields (Braun and Sutton, 1987). The relationships between necrotic tissue present in the crop, flower infection and subsequent fruit rot have already been documented in that system (Jordan, 1978; Braun and Sutton, 1987). In annual strawberry crops using waiting-bed plants, however, the epidemiology may differ. It seems that the amount of necrotic tissue varies with the quality of the transplants and such necrotic tissue does not determine the inoculum pressure of *B. cinerea* for flower colonisation (Chapter 2).

Biological control of strawberry grey mould can be achieved by interfering with the infection process of the pathogen at the blossom stage and during development of fruits (Bhatt and Vaughan, 1963), for example by using yeast as antagonists (Lima et al, 1997). Bhatt and Vaughan (1963) studied biocontrol of grey mould in strawberry by *Cladosporium* spp. The effect of the fungus on the reduction of fruit rot at harvest

was attributed to the prevention of establishment of *B. cinerea* on the senescent and dead flower organs of strawberry. Another approach, which consists of suppression of *B. cinerea* inoculum production by *Gliocladium roseum*, was reported recently (Peng and Sutton, 1991). Jordan and Pappas (1977) suggested a similar approach, suppression of inoculum production, by chemical means. In perennial crops, grey mould control of strawberry by reducing initial inoculum production on necrotic leaves within the crop is likely to be effective. Infection of most strawberry flowers takes place in a single infection cycle by conidia produced on necrotic leaves present in the crop during flowering (Sutton, 1990b). For the annual cropping system using waiting-bed transplants, such inoculum may play a different role so that biocontrol strategies need to be adjusted.

A strong fungal competitor in necrotic tissue, *Ulocladium atrum*, was found in bioassays to be more effective to exclude *B. cinerea* on dead lily leaves than other antagonists such as *Gliocladium catenulatum, Aureobasidium pullulans* or *Chaetomium globosum* (Köhl et al, 1995a). For several other crops, such as cyclamen (Köhl et al, 1998), tomato (Fruit and Nicot, 1999) and grapevine (Schoene and Köhl, 1999), it was demonstrated that *U. atrum* can successfully compete with *B. cinerea* resulting in effective disease control. According to Cook (1993), the effectiveness of a competitive biological control agent used in an inundative release method depends on inoculum density, frequency of application and the application technique as well as micro-climatic conditions. The effect of inundative release of *U. atrum* on grey mould of strawberry has not been tested yet under field conditions.

The objective of our study was to evaluate under field conditions the effectiveness of *U. atrum* in reducing strawberry fruit rot caused by *B. cinerea* in annual strawberry crops with waiting-bed transplants. Attempts were made to optimise timing, initial applications, spraying frequency and suspension concentration.

MATERIALS AND METHODS

Experimental set-up

Eight field experiments were conducted with annual strawberry crops, using waiting-bed transplants, cv. Elsanta, produced in the previous year and cold-stored at -2 °C until the day before planting. All experiments were carried out in a completely randomised block design with four to six blocks (Table 1). Experiments 1, 4, and 7 were located in Breda, the Netherlands. There were 40 plants per plot in four single rows of ten plants per row and the distance between rows was 0.8 m. Experiments 1 and 4 were established inside a field of red cabbage serving as buffer crop with a distance of 10 m between plots. Experiment 7 had no buffer crop between the plots.

Experiments 2, 3, 5, 6, and 8 were located on a sandy soil near Wageningen, the Netherlands (Table 1). The plots were established within a grass field, separated by 10 m of grass buffer. Each plot consisted of 78 plants in three twin rows of 2 x 13 plants per twin row. The distance between twin rows was 1 m, and within twin rows 0.5 m, having three plants per m row length.

Table 1. General description of strawberry experiments, the Netherlands.

Exp. number	Location	Previous crop	Transplanting date	Number of replicates	Harvesting period
1	Breda	Strawberry	2 July 1996	6	29-Aug to 4-Sep 1996
2	Wageningen	Grass	10 April 1997	5	16-Jun to 17-Jul 1997
3	Wageningen	Grass	27 June 1997	5	11-Aug to 25-Aug 1997
4	Breda	Strawberry	25 June 1997	6	14-Aug to 26-Aug 1997
5	Wageningen	Grass	6 May 1998	4	02-Jul to 03-Aug 1998
6	Wageningen	Grass	19 June 1998	4	13-Aug to 07-Sep 1998
7	Breda	Strawberry	29 April 1998	4	25-Jun to 21-Jul 1998
8	Wageningen	Grass	21 May 1999	4	15-Jul to 09-Aug 1999

Fungal inoculum

Fresh conidial suspensions of *U. atrum*, isolate 385, were prepared from four week old cultures of the antagonist grown on oat grains (Köhl et al, 1995a). Conidia were

washed from the substrate in a nylon bag using a camping washing machine (Miniwash Nova, Belgium) filled with water containing 0.01% Tween 80. The resulting suspension was filtered through a nylon gauze (200 μ mesh). The concentration was determined using a haemocytometer and the suspension was adjusted to the proper concentration for each treatment. Suspensions were stored at 4 °C until use, which took place within 24 h or 48 h after preparation of the suspension in experiments at Wageningen and Breda, respectively.

Treatments

The control treatment was an unsprayed plot except in experiment 1, where water containing Tween-80 at 0.01% (v/v) was applied weekly from transplanting until fruit colour was beginning to change to red.

U. atrum was applied at a concentration of 2 x 10^6 conidia per ml, except for experiment 7, where the concentration was also object of study, and experiment 8, where the concentration was 0.5 x 10^6 conidia per ml. The *U. atrum* application programmes started either from transplanting (*U. atrum* - transplanting) or from opening of the first flower (*U. atrum* - flowering) and stopped when the first fruit changed colour to red. The frequency in *U. atrum* spray programmes was weekly in experiments 1, 2, 3 and 4 changing to twice weekly in treatment 2 at first green bud appearance; weekly from transplanting and twice weekly from first flower opening in experiments 5 and 6; weekly or twice weekly from first flower opening, in experiment 7; and every second or fourth day from first flower opening in experiment 8.

Fungicide programmes started from first open flower till first fruit colour change to red. Sprays were applied weekly in experiments 1, 2, 3, 4 and 7; and twice weekly in experiments 5, 6 and 8.

Crop sanitation treatments were carried out only in experiments 5 and 6, and consisted of removal of all senescing leaflets from the strawberry plants twice per week, starting immediately after transplanting till first fruit colour change to red.

Experiment 1 had the treatments (1) Control - (water+Tween-80; 8 sprays); (2) *U. atrum* - transplanting (8 sprays); (3) Fungicides - (4 sprays) one application of iprodione (1.5 kg/ha of Rovral wp® at 50%, Rohm and Haas, France), two applications of tolylfluanide (1 kg/ha of Euparene M® at 50%, Bayer, the Netherlands)

and one application of pyrazophos (0.5 litre per ha of Curamil® at 30%, Hoechst Schering AgrEvo, the Netherlands).

Experiments 2 and 3 had the treatments (1) Control; (2) *U. atrum* - transplanting (11 sprays in exp. 2; 6 sprays in exp. 3); (3) *U. atrum* - flowering (5 sprays in exp. 2; 3 sprays in exp. 3); (4) Fungicides - alternating Thiram (2 kg/ha of TMTD ® at 98%, Satec, Germany), tolylfluanide (0.75 kg a.i./ha, 3 sprays in exp. 2; 1 spray in exp. 3) and pyrimethanil (2 litre per ha of Scala® at 40%, AgrEvo, the Netherlands).

Experiment 4 had the treatments (1) Control; (2) *U. atrum* - transplanting (6 sprays); (3) *U. atrum* - flowering (3 sprays); (4) Fungicides - (4 sprays) alternated applications of tolylfluanide (0.75 kg a.i./ha) and iprodione (0.75 kg a.i./ha).

Experiments 5 and 6 had the treatments (1) Control; (2) *U. atrum* - transplanting (12 sprays in exp. 5; 8 sprays in exp. 6); (3) *U. atrum* - flowering (6 sprays in exp. 5; 5 sprays in exp. 6); (4) Fungicides - alternated sprays of tolylfluanide (0.75 kg a.i./ha, 3 sprays for both experiments) and iprodione (0.75 kg a.i./ha, 3 sprays in exp. 5; 2 sprays in exp. 6); (5) Crop sanitation.

Experiment 7 had the treatments (1) Control ; (2) - (4) *U. atrum* - weekly at 0.5 x 10^6 conidia per ml, 1 x 10^6 conidia per ml and 2 x 10^6 conidia per ml, respectively (4 sprays); (5) - (7) *U. atrum* - twice weekly at 0.5 x 10^6 conidia per ml, 1 x 10^6 conidia per ml and 2 x 10^6 conidia per ml, respectively (7 sprays); (8) Fungicides - alternating iprodione (0.75 kg a.i./ha) and tolylfluanide (0.75 kg a.i./ha) (4 sprays).

Experiment 8 had the treatments (1) Control; (2) *U. atrum* - flowering, every second day (11 sprays); (3) *U. atrum* - flowering, every fourth day (6 sprays); (4) Fungicides - tolylfluanide (0.75 kg a.i./ha) and iprodione (0.75 kg a.i./ha) (3 sprays of each fungicide); (5) Water - every second day spraying with 0.01% of Tween 80 (11 sprays).

The sprays at Breda (exp. 1, 4, and 7) were applied with a knapsack sprayer using a hollow-cone nozzle and air-pressure at 200 kPa, with an application rate of 500 litre per ha. Sprays in the experiments at Wageningen (exp. 2, 3, 5, 6, and 8) were applied with a propane gas-driven knapsack sprayer at 250 kPa and application rate of 750 litre per ha.

Crop management and cultural practices

In all experiments except experiment 7, interval overhead irrigation was applied during periods of hot and dry weather in order to promote development of *B. cinerea*. Weeding was done mechanically. The soil was covered with straw from beginning of flowering. No insecticides, herbicides, or other fungicides were applied except for experiment 1 where deltametrin was applied three times for thrips control and experiment 4 where transplants had been treated with a fungicide (fosetyl-Al plus benomyl) according to normal recommendations.

Incidence of *B. cinerea* on flowers

In experiments 2, 3, 5, and 6, samples of 20 to 30 flowers, each flower from a different arbitrarily chosen plant, were collected per plot at each of two or three sampling dates. In experiment 8, 50 petals, each petal from a different plant, were collected per plot instead of flowers. The flowers or petals were harvested at flower maturity when the anthers appeared brown and the petals started to fall. Flowers were put into plastic boxes (22 cm length x 14 cm width x 5 cm height) with wet filter paper on the bottom and incubated at 18 °C for 14 days in the dark. Petals were incubated under the same conditions in sterile plastic petri dishes (14 cm ∅ x 2 cm height), containing moist filter paper on the bottom. After incubation, the presence of sporulation of *B. cinerea* was assessed on the flower parts, viz. sepals, petals, stamens and pistils, with a stereomicroscope, at 10-100 x magnification (Olympus Optical Co, Japan). The incidence of *B. cinerea* sporulation on flowers or petals was calculated per plot.

Harvest and post-harvest assessments

Fruits were picked twice per week. Ripe symptomless fruits were harvested first and kept separate from harvested diseased fruits. Fruits with symptoms were also picked when still in the white stage. Healthy and diseased fruits per plot were weighed and counted.

Fruit rot

In experiments 2, 3, 5, 6, and 8, the ripe and white diseased fruits were assessed for characteristic symptoms caused by *B. cinerea, Colletotrichum acutatum* or *Zythia fragariae* (teleomorph *Gnomonia comari* Karsten). In experiments 1, 4 and 7 symptoms were classified as those of *B. cinerea* and other diseases without further differentiation. Fruits with non-specific symptoms were put without contact among fruits into moist chambers consisting of a plastic tray (50 cm length x 30 cm width x 7 cm height) with wet paper on the bottom. The tray was sealed with a plastic bag, incubated at 20 °C for two days in the dark and reassessed. Fruits with characteristic symptoms were then added to the corresponding disease category, and fruits with non-specific symptoms were classified as "other diseases".

Post-harvest fruit rot from a sub-sample of symptomless fruits was assessed for experiments 1, 2, 3, 5, and 6. Twenty arbitrarily chosen fruits per plot were put into a plastic tray (50 cm length x 30 cm width x 7 cm height) with wet paper on the bottom without contact among fruits, closed with a plastic bag and stored at 4 °C during five days in the dark followed by three days incubation at 20 °C. Thereafter, fruits were assessed for disease incidence namely symptoms of *B. cinerea.*

Data analysis

The statistical analysis was performed by analysis of variance, ANOVA, followed by LSD-tests (Snedecor and Cochran, 1989) using Genstat 5 version 4.1 (Numerical Algorithmic Group Inc., Oxford, UK). The ANOVA and LSD-test for percentage of grey mould was done after angular transformation, due to low values of this variable. Data are reported in the text as means per treatment and experiment. A complementary factorial analysis was done on that part of the data of grey mould incidence of experiment 7, that constituted a factorial experiment with spraying interval as one factor and three levels of conidial concentration as the other factor.

RESULTS

Grey mould at harvest

The incidence of grey mould in untreated plots was generally low, from 1.4 to 12.3% of the total fruits (Tables 2-4). The application of *U. atrum* weekly at 2 x 10^6 conidia per ml from transplanting till first red fruit significantly reduced the percentage of grey mould in comparison to the control in experiments 1, 2 and 4 (Table 2). *U. atrum* applied weekly from the beginning of flowering at 2 x 10^6 conidia per ml, gave significant reduction of grey mould in comparison to the control in experiment 2 and experiment 7 but not in experiment 4 (Tables 2 and 4). *U. atrum* weekly sprayed from transplanting and twice weekly from beginning of flowering, significantly reduced grey mould in comparison to the control in experiment 5, but not in experiment 6 (Table 3). *U. atrum* at 2 x 10^6 conidia per ml, applied twice per week from the beginning of flowering, reduced grey mould in comparison to the control in experiment 5 (Table 3) and experiment 7 (Table 4) but not in experiment 6 (Table 3). In experiment 7 (Table 4), applications with 0.5, 1 or 2 x 10^6 conidia per ml of *U. atrum*, twice per week from the beginning of flowering till first red fruit, significantly reduced grey mould in comparison to the control. However, when *U. atrum* was applied weekly, only conidial suspensions with 2 x 10^6 conidia per ml, significantly reduced grey mould in comparison to the control. In experiment 8 (Table 4), *U. atrum* applied at 0.5 x 10^6 conidia per ml from the beginning of flowering till first red fruit, did not reduce grey mould in comparison to the control when applied at two or four days intervals. However, the incidence of grey mould was low with 2.2% in the control treatment.

In experiment 3, no treatment effect on grey mould could be detected but grey mould incidence was below 2% in the control. The effects of applications of *U. atrum* at 2 x 10^6 conidia per ml starting at transplanting were superior to those starting at flowering in only one experiment (exp. 4, Table 2). Analysis of variance of *U. atrum* treatments during flowering, at three concentration levels once every week or every two weeks (exp. 7), showed that irrespective of the conidial concentration, twice weekly sprays yielded significantly less grey mould than weekly sprays of antagonist (F-test; $P<0.008$). *U. atrum* did not lose efficacy in reducing grey mould when conidial concentration was lowered from 2 x 10^6 conidia per ml to 0.5 x 10^6 conidia per ml.

Fungicide programmes reduced grey mould significantly more than *U. atrum* applied at 2 x 10^6 conidia per ml from the beginning of flowering, in five experiments (exp. 2, 4, Table 2; exp. 5, 6, Table 3; exp.7, Table 4).

Crop sanitation by removing dead leaves did not reduce grey mould in comparison to the control (exp. 5 and exp. 6, Table 3).

Thus, *U. atrum* spray programmes effectively reduced grey mould in five of eight experiments. In two of eight experiments at least one *U. atrum* spray programme gave the same or better control than the fungicide programme (exp. 1 and 4, Table 2). In experiment 3, the lack of significance can be explained by the extremely low incidence of fruit rot (1.4% in the control) due to dry conditions.

Post-harvest grey mould

Grey mould in the control treatment at post-harvest (8.8 to 35%) was higher than at harvest (1.4 to 12%) and in two of five experiments treatment effects were found. *U. atrum* spray programmes either from transplanting or from the beginning of flowering reduced post-harvest grey mould in comparison to the control only in one (exp. 2) of five experiments (exp. 1-3 and 5,6; Tables 2 and 3). The fungicide programme reduced post-harvest grey mould better than the *U. atrum*-spraying programme only in one experiment. Removal of dead leaflets from strawberry plants (crop sanitation) did not reduce grey mould at post-harvest in comparison to the control (exp. 5 and 6, Table 3).

Table 2. Effect of applications of conidial suspensions of a *Ulocladium atrum* or fungicide programme on *Botrytis* fruit rot at harvest and post-harvest. Field experiment one was carried out in 1996 in Breda, experiment 2 and 3 in 1997 in Wageningen, and experiment 4 in 1997 in Breda, the Netherlands.

Treatments	*Botrytis* fruit rot at harvest (%)[x]				*Botrytis* fruit rot at post-harvest (%)[x]				Number of sprays			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4
(1) Control untreated[y]	12.3 a	5.44 a	1.43	8.05 a	38	16.2 a	21.2	-	8	0	0	0
(2) *U.atrum*_weekly (starting at transplanting)[z]	4.8 b	2.63 b	1.08	4.72 b	30	10.3 b	17.6	-	8	11	6	6
(3) *U. atrum*_weekly (starting at beginning flowering)[z]	-	3.42 b	2.02	7.09 a	-	7.78 b	21.2	-	-	5	3	3
(4) Fungicides_weekly (starting at beginning flowering)	11.5 a	1.27 c	1.48	3.91 b	37	5.67 b	15.8	-	4	5	3	4

[x] Values in the same column followed by the same letter are not significantly different (LSD-test; $P<0.05$).

[y] The control for experiment 1 was sprayed with water plus Tween-80 at 0.01%.

[z] *U. atrum* was applied at weekly intervals with 2×10^6 conidia per ml.

Table 3. Effect of applications of conidial suspensions of *U. atrum* or fungicide programme on grey mould at harvest and post-harvest. The experiments were carried out in 1998, Wageningen, the Netherlands.

Treatments	Grey mould at harvest (Field fruit rot) (%)[x]		Grey mould at post-harvest (%)[x]		Number of sprays	
	Exp. 5	Exp. 6	Exp. 5	Exp. 6	Exp. 5	Exp. 6
(1) Control untreated	8.25 a	11.32 a b	8.8 a	33.2 a	-	-
(2) *U. atrum* (starting at transplanting)[y]	5.85 b	8.78 b	14.9 a	29.3 a	12	8
(3) *U. atrum* (starting at beginning flowering)[z]	5.61 b	10.59 b	6.9 a	26.8 a	6	5
(4) Fungicide	3.63 c	3.0 c	7.5 a	19.3 b	6	5
(5) Leaf-Sanitation	8.33 a	13.91 a	8.8 a	31.4 a	-	-

[x] Values in the same column followed by the same letter are not significantly different (LSD; $P<0.05$).

[y] *U. atrum* was applied weekly from transplanting and twice weekly from first green bud appearance with 2 x 10^6 conidia per ml .

[z] *U. atrum* was applied twice weekly from first open flowers, with 2 x 10^6 conidia per ml.

Table 4. Effect of application intervals of conidial suspensions of *U. atrum* at flowering time or fungicide programme on grey mould at harvest. (A) Field experiment 7, in Breda, 1998, and (B) field experiment 8, in Wageningen, 1999, the Netherlands.

Treatments	Grey mould at harvest (%)[x]
(A) Experiment 7	
(1) Control (untreated)	2.8 a
(2) *U.atrum* weekly; 0.5 x 10^6 conidia per ml	2.5 a b
(3) *U.atrum* weekly; 1 x 10^6 conidia per ml	2.4 a b c
(4) *U.atrum* weekly; 2 x 10^6 conidia per ml	1.8 b c d
(5) U.atrum twice per week; 0.5 x 10^6 conidia per ml	1.6 c d
(6) U.atrum twice per week; 1 x 10^6 conidia per ml	1.4 d
(7) U.atrum twice per week; 2 x 10^6 conidia per ml	1.6 c d
(8) Fungicide (weekly)	0.7 *e*
(B) Experiment 8	
(1) Control (untreated)	2.2 a
(2) *U. atrum* 2 days intervals[y]	1.7 a
(3) *U. atrum* 4 days intervals[y]	1.4 a
(4) Fungicide (twice per week)	0.5 b
(5) Water + Tween 80; 2 days intervals	1.5 a

[x] Values in the same column followed by the same letter are not significantly different (LSD-test of angular-transformed values; $P<0.05$).

[y] With 0.5 x 10^6 conidia per ml from first flower opened.

Incidence of *B. cinerea* on flowers and petals

The incidence of *B. cinerea* on flowers (Table 5) from plants treated with *U. atrum* conidia starting at transplanting time was lower than the control in sample 2, experiment 2 (sampled 3 days after spraying), in sample 1, experiment 5 (sampled 3 days after spraying) and in sample 2, experiment 5 (sampled 4 days after spraying). Fungicide treatment resulted in a lower incidence of *B. cinerea* on flowers than the control in sample 2, experiment 5 (sampled 4 days after spraying) and sample 3, experiment 5 (sampled 3 days after spraying). Only in sample 2, experiment 5 (1998) the fungicide programme resulted in a lower incidence of *B. cinerea* in flowers as compared to flowers treated *with U. atrum* sprayed from beginning of flowering (sampled 4 days after spraying).

In experiment 3 (1997) and experiment 6 (1998) no differences between treatments were found for the incidence of *B. cinerea* on flowers. In experiment 8, the incidence of *B. cinerea* on petals from plots treated with *U. atrum* or fungicides was lower as compared to untreated plots. However, these differences were statistically significant only for sample 1. The incidence of *B. cinerea* on petals did not differ between the untreated plots and plots with crop sanitation (exp. 5, 6).

Table 5. Effect of applications of conidial suspensions of *U. atrum*, fungicide programmes or crop sanitation on the incidence of *B. cinerea* on flowers (experiment 2, 3, 5, and 6) and on petals (experiment 8), Wageningen, the Netherlands.

Experiment	Interval between the previous spray and sampling day	Incidence of *B. cinerea* on flower / petals[x] (1) Control untreated	(2) *U. atrum* (from transplanting)	(3) *U. atrum* (from beginning of flowering)	(4) Fungicide (from beginning of flowering)	(5) Crop sanitation[z]
Experiment 2 (1997)						
-sample 1 (10-Jun)	5	5.1 a	3.0 a	3.0 a	10.0 a	-
-sample 2 (16-Jun)	3	14.9 a	1.3 b	1.3 b	6.0 a b	-
Experiment 3 (1997)						
-sample 1 (29-Jul)	7	76.2 a	87.0 a	93.8 a	83.0 a	-
-sample 2 (4-Aug)	6	96.0 a	92.0 a	90.0 a	97.0 a	-
Experiment 5 (1998)						
-sample 1 (08-Jun)	3 (only treat 2)[y]	63.7 a	33.7 b	83.7 a	62.5 a	65.0 a
-sample 2 (15-Jun)	4	70.0 a	42.5 b c	51.2 a	21.3 c	62.5 a b
-sample 3 (03-Jul)	3	81.3 a	66.2 a b	64.0 a b	51.8 b	72.5 a
Experiment 6 (1998)						
-sample 1 (21-Jul)	5 (only treat 2)[y]	83.7 a	72.5 a	83.7 a	81.2 a	80.0 a
-sample 2 (5-Aug)	1	75.0 a	53.7 a	63.7 a	53.7 a	67.5 a
-sample 3 (11-Aug)	4	60.1 a	51.2 a	53.7 a	55.0 a	66.2 a
Experiment 8 (1999)		(control, untreated)	(*U. atrum*, 2 days intervals)	(*U. atrum*, 4 days intervals)	(Fungicide, 4 days intervals)	(Water+Tween, 2 days intervals)
-sample 1 (28-Jun)	2	45.0 a b	33.5 b c	25.0 c	24.0 c	50.5 a
-sample 2 (30-Jun)	2(treat 2), 4(treat 3, 4)	18.8 a	5.0 a	5.5 a	12.0 a	17.0 a

[x] Values in the same row followed by the same letter are not significantly different (LSD-test; P<0.05).
[y] In the sample 1 (exp. 5 and exp. 6), only treatment 2 had been sprayed before this sampling date.
[z] Removal of senescent leaflets twice per week from transplanting till first fruit colour change to red.

Fruit quality

Healthy fruit weight, as measure of quality, did not differ among the treatments, except in experiment 5 where plants treated twice weekly with *U. atrum* at 2 x 10^6 conidia per ml from first flowering presented significantly smaller fruits (8.8 g per fruit) than the control (10.3 g per fruit). In experiment 8, plants sprayed with fungicide presented significantly higher fruit weight (13.2 g) than the control (11.6 g) (other data not presented).

DISCUSSION

U. atrum was effective in reducing grey mould at harvest at least in one spray programme per experiment in five out of eight experiments. In the experiments 3 and 8, *U. atrum* failed to show an effect on grey mould probably due to the low incidence of grey mould with 1.4 and 2.2%, respectively. In combination with hot conditions overhead (exp. 3) or mist (exp. 8) irrigation systems were inadequate to stimulate grey mould incidence in these experiments.

Additional sprays of *U. atrum* before flowering, starting from transplanting compared to spraying during flowering only, resulted in an improved control level of grey mould at harvest only in one out of five experiments. Bhatt and Vaughan (1962) studied the biocontrol of grey mould in strawberry by *Cladosporium* spp. The effect of the fungus on the reduction of fruit rot at harvest was attributed to prevention of establishment of *B. cinerea* on the senescent and dead flower organs of strawberry. The same mechanism may be involved in the interaction between *U. atrum* and *B. cinerea*. In this case, targeting applications to flower parts would be effective to reduce *B. cinerea* infection on annual strawberry crop. Moreover the small amount of necrotic leaves produced inside annual strawberry crops, may mean that the inoculum produced on these tissues does not play an important role in the epidemiology of grey mould in such cropping systems (Chapter 2).

This explains the limited additional effect of *U. atrum* treatments before flowering, although the antagonist is able to colonise and suppress the sporulation of *B. cinerea* on dead strawberry leaves (Chapter 2). Crop sanitation by removing

senescent leaves in our system did not reduce *Botrytis* infection as it did in similar experiments in onion crops (Köhl et al, 1995b).

Shorter application intervals of *U. atrum* resulted in better control even when lower concentrations were applied (exp. 7, Table 4). In the strawberry crops new flowers are produced each day and flower parts start to senesce after three to five days. Since the mode of action of *U. atrum* is by substrate competition the antagonist should reach the site of colonisation on the flower parts before or at the same time as the pathogen (Köhl, 1995a; Kessel, 1999). This explains the need of frequent applications to reach all flowers at an early stage.

The conidial concentration can be reduced down to 0.5 x 10^6 conidia per ml without losing the efficacy of *U. atrum* to control grey mould at harvest, when sprayed twice per week. *U. atrum* tested under field conditions showed its potential to control grey mould at harvest by spraying conidia from transplanting or either once and twice weekly during flowering only. Further studies should evaluate the performance of the antagonist on strawberry crops under conditions more favourable to grey mould development.

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Chapter 4

CONIDIAL PERSISTENCE AND COMPETITIVE ABILITY OF THE ANTAGONIST *ULOCLADIUM ATRUM* ON STRAWBERRY LEAVES

Abstract

The persistence of conidia over time, and competitive ability of *Botrytis cinerea* antagonist *Ulocladium atrum* were studied in field plots of strawberry in the Netherlands. Conidial suspensions of the antagonist were sprayed on the canopy in 14 applications during spring, summer and early winter of 1997 and 1998. Regular leaf samples were taken up to 120 days after application to assess total conidial density, conidial viability, and competitive colonisation of necrotic leaf tissue by *U. atrum* vis-à-vis naturally occurring *B. cinerea* and other necrotrophic fungi. The density of *U. atrum* conidia on green strawberry leaves declined exponentially after application, on average with a relative rate of decrease of -0.10 day^{-1}. Variation in the rate of decrease between individual sprays was large and partly associated with the average daily precipitation during the first week after application. Percentage viability of *U. atrum* conidia declined only slowly over time after application. For the pooled data, a linear decline at a rate of 1% day^{-1} was found. Colonisation of necrotic strawberry leaf tissue by *U. atrum*, increased with conidial density, and at densities higher than 1500 conidia cm^{-2} leaf colonisation by naturally occurring *B. cinerea* was consistently reduced. With the current level of persistence, effective suppression *of B. cinerea* on strawberry leaves can only be expected when *U. atrum* is applied less than a week before the leaves become necrotic. Improved persistence of *U. atrum* conidia on the leaf surface will reduce the need for frequent applications, and it is therefore suggested that research in this area should focus on enhanced rain-fastness of deposited conidia to reduce spore loss.

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INTRODUCTION

Botrytis cinerea Pers. is an important pathogen causing diseases in several crops, including grey mould on strawberries (Jarvis, 1980b; Maas, 1984). Braun and Sutton (1987) studied grey mould epidemiology in perennial strawberry systems and demonstrated that the pathogen *B. cinerea* produces conidial inoculum mainly on crop debris inside the field. Suppression of inoculum production of *B. cinerea* on this source by using antagonists such as *Gliocladium roseum* effectively controlled grey mould on fruits (Sutton, 1990a; Sutton and Peng, 1993). The use of a biological control agent to suppress fungal sporulation has a potential advantage over suppressing infection, because the interaction may take place over a much longer period (Fokkema, 1993). This approach was also tested with the fungal antagonist *Ulocladium atrum*, which provided effective control of *B. cinerea* in greenhouse crops of pot roses (Köhl and Gerlagh, 1999), cyclamen (Köhl et al, 2000), and geranium (Gerlagh et al, submitted). In field experiments *U. atrum* also reduced grey mould significantly in grapevine (Schoene and Köhl, 1999) and strawberry (Chapter 3). In annual strawberry crops, several experiments indicated that applying *U. atrum* to the strawberry canopy before flowering provided an additional reduction of grey mould, as compared to sprays of *U. atrum* starting at beginning of flowering (Chapter 3). Thus, strawberry is a crop where *U. atrum* is a potential biocontrol agent of *B. cinerea*.

The saprophytic fungus *U. atrum* affects *B. cinerea* by competitive colonisation of the necrotic tissue which the pathogen uses as inoculum source or entry to healthy tissue (Köhl et al, 1997; Kessel, 1999). Thus, the effectiveness of *U. atrum* will be enhanced if the antagonist remains present and viable, once introduced into the canopy at an early stage of development of the plant. In this way, colonisation could start as soon as the tissue becomes senescent and dies (Tronsmo, 1992). Studies by Elmer and Köhl (1998) on survival of *U. atrum* in lily canopies showed that the ability of the antagonist to compete with naturally occurring saprophytes lasted until at least 21 days after spraying. Suppression of *B. cinerea* could not be measured because this pathogen was absent in their experiment. Moreover, the persistence, i.e. the density of viable spores over time, and competitive ability of *U. atrum* conidia in the

strawberry crop may differ from those in lily or other crops, because of differences in e.g. canopy structure, leaf surface properties, microbial diversity in the phyllosphere and composition of the leaf tissue. For over-wintering and perennial strawberry crops, the ability of *U. atrum* conidia to survive through winter is also of interest.

The research described in this paper was conducted to quantify the persistence of *U. atrum* conidia applied on strawberry leaf tissue, and the competitive colonisation of this tissue by the antagonist with respect to the suppression of *B. cinerea* sporulation. The results will be helpful to determine appropriate timing of sprays in relation to tissue senesce and to assess the possible need for improvement of the persistence on the applied conidia on the target tissue.

MATERIALS AND METHODS

Field experiments

Four experiments were carried out in small fields plots of strawberry cv. Elsanta, on a sandy soil near Wageningen, the Netherlands. The transplanting dates were (1) 6 May 1998; (2) 19 June 1998; (3) 8 September 1997 and (4) 21 August 1998. Plants for experiments 3 and 4 were left in the field during wintertime. The strawberry plots consisted of 156 plants in an area of 4.5 x 8.66 m surrounded by grass. The distance between the plots was 10 m. Cold-stored transplants, kept at –2 °C until the day before planting, were transplanted in double rows with 3 plants per meter row length. Row spacing was 1 m between double rows and 0.5 m within double rows. Mist irrigation (experiments 1, 2 and 4) and sprinkler irrigation (experiment 3) were used to ensure water supply for plant development during dry periods. No insecticides or herbicides were sprayed and weeding was done manually.

Microclimate

Weather variables were monitored in an extra strawberry plot at 20 m distance from the experiment, in which irrigation and cultural management were identical to those in the experiments. Relative humidity and temperature were monitored with an electronic sensor (Pow 8-35 VDG, Rotronic AG, Bassersdorf, Switzerland) positioned

at 30 cm height within the strawberry plot. Precipitation including irrigation was measured by a recording rain gauge (Casella, London, UK). Wetness of necrotic leaves was measured in the canopy at 5 cm height, with leaf wetness sensors developed at Plant Research International (Köhl et al, 1995a). All data were stored each half-hour by a data-logger (Delta-T Devices Ltd, Cambridge, UK).

U. atrum inoculum production and field applications

For all experiments fresh conidial suspensions of *U. atrum* were prepared on each spraying day. Conidia from a four week old culture of *U. atrum* grown on oat grains were washed from the substrate using a camping washing machine filled with tap water containing 0.01% Tween 80 (Köhl et al, 1995a). The resulting suspension was filtered through nylon gauze (200-μ mesh). The concentration was determined using a haemocytometer and adjusted to 2×10^6 conidia ml^{-1} before spraying. Four (exp. 1 and 2) or three strawberry plots (exp. 3 and 4) were sprayed once with the conidial suspension. The sprayed plot consisted of a set of ten plants in double rows per spraying time and was paired by a control plot of the same size in experiments 3 and 4. For each application, the potential germination of *U. atrum* conidia in the suspension was checked on water-agar and on sprayed green strawberry leaves, after 12 hours of incubation at 18 °C in the dark. Sprays were applied on 7 May, 13 May, 3 June and 9 June 1998 for experiment 1; 23 June, 28 June, 16 July and 22 July 1998 for experiment 2; 4 Nov, 2 Dec and 19 Dec 1997 for experiment 3; 19 Nov 1998, 3 Mar 1999 and 23 Apr 1999 for experiment 4. All sprays were applied at 250 kPa, at rate of 750 l ha^{-1}, with an air-driven knapsack sprayer held at 20 cm distance from the foliage. Fully expanded green leaves were labelled before spraying. Necrotic leaves at the time of spraying were separately labelled. Inoculum of *B. cinerea* was not artificially introduced in any of the experiments.

Leaf sampling

Samples of five to six labelled green leaflets were randomly harvested, each from a different plant, in unsprayed and sprayed plots per sampling date several times after spraying, as shown in Figure 1 and 2. Sampling started from one hour after spraying for the applications on 13 May 1998, 3 June 1998 (exp. 1); 16 July 1998 (exp. 2); 2

December 1997, 19 December 1997 (exp. 3); 19 November 1998 and 3 March 1999 (exp. 4). For sprays on 7 May 1998, 9 June 1998 (exp. 1); 23 June 1998, 28 June 1998, 22 July 1998 (exp. 2); 4 November 1997 (exp. 3); 29 April 1998 (exp. 4), the first sample was taken 24 hours after spraying. Leaves that were necrotic at the time of spraying were sampled only for the first evaluation one or 24 hours after spraying. Subsequently, necrotic leaves were sampled from leaves that were green at the time of spraying. These leaves were always located nearby the soil and older than those still green. In experiment 3, naturally dead leaves were not sampled because few were present. Sampled leaflets, green or necrotic, were immediately processed in the lab. Three discs (Ø = 1.4 cm) were prepared with a cork-borer from each leaflet to assess fungal colonisation; the remaining leaflet tissue was used for determining conidial survival. Thus, the same leaflets per evaluation date, per spray, were used to study both survival of *U. atrum* spores and colonisation. Attempts to assess survival of *U. atrum* conidia on naturally necrotic tissue failed due to the presence of dust and sporulation of various other fungi.

Survival of the antagonist

Survival of *U. atrum* was assessed by measuring the density and viability of conidia on sprayed and unsprayed green leaflets. Leaflets were placed separately into a plastic petri dish with wet paper on the bottom and incubated for 12 hours at 18 °C in the dark, to allow *U. atrum* conidia to germinate. After that, two pieces of leaf tissue of about 1.0 cm^2, one from the top and another from the bottom of each leaflet were placed on a glass slide. A drop of fluorochrome calcofluor white, CF (Fluorescent brightness 28, Sigma; 0.02% w/v in 1M tris HCl buffer, pH 8.0) was added to the leaf surface and mixed with another drop of fluorescein di-acetate, FDA stain (F-7378, Sigma; 80 ppm w/v in phosphate buffered saline, pH 7.2). A cover glass was then placed over the drop containing both stains. All conidia in ten fields of 1 mm^2 were counted using a fluorescence microscope (Zeiss Axioskop, Germany). Spore germination (stained with CF) was examined with filter 05 and spore viability (stained with FDA) with filter 09. A conidium was considered germinated (or viable) if at least one stained germ tube longer than half of a conidium diameter emerged from the *U. atrum* conidium. The data from the two pieces of leaflet were averaged for statistical

analysis as a single replicate. For each sampling date, also normalised values for spore density and spore viability were calculated by taking the ratio of the actual value and the value of spore density or percentage of viability at first evaluation.

Fungal colonisation

Discs of green and naturally necrotic leaf tissue were taken at the same time from sprayed and unsprayed plants to determine potential fungal sporulation before processing the sample to assess conidium survival. Three discs of 1.54 cm^2 per leaflet (replicates), from the top, middle and bottom, were put into a plastic petri dish with water/agar for naturally necrotic tissue and with water/agar + paraquat, at 10 mg l^{-1} (Gramoxone 200, Zeneca, UK) for green leaf tissue. The paraquat was added to the media to induce necrosis and to allow colonisation of *U. atrum*, at a concentration that not affect the antagonist (Elmer and Köhl, 1998). Petri dishes with green and naturally necrotic tissue discs were sealed in plastic bags and incubated for 14 days at 18 °C in the dark. After incubation, the area with sporulation of *U. atrum*, *B. cinerea*, *Alternaria* spp, *Cladosporium* spp, *Zythia fragariae* and other saprophytic fungi was assessed with the aid of a stereo microscope (Olympus optical Co, Japan) and expressed as a percentage of the total leaf disc area. The average of the three discs, representing a leaflet sample, was used for statistical analysis.

Data analysis

Linear and non-linear regression analysis were performed with Genstat 5 version 4.1 (Numerical Algorithm Group, Inc., Oxford, UK). Non-linear regression analysis was performed with the normalised spore density data of *U. atrum*. Linear regression analysis was performed with the normalised spore viability data of *U. atrum*. Models for each analysis were fitted to the data of each individual spray of four experiments and to the pooled data obtained until 70 days after the first evaluation. Conidial densities of *U. atrum* on green leaflets were also used as independent variables in the analysis of potential fungal sporulation on leaf discs.

Pearson's (r) and Spearman's (r_s) coefficient of correlation were calculated between the rates of decrease in spore density an viability of *U. atrum* and the weather variables in experiments 1 and 2 (Snedecor and Cochran, 1989). Data from

experiments 3 and 4 were not included in the correlation analysis because the evaluation interval for most sprays was too large. For three consecutive periods of six to seven days after spraying, a relative daily rate of decrease in spore density was calculated by dividing the change in density over the period by the density at the start of the period and the length of the period in days (6 or 7). For each period, also the rate of decrease in spore viability was calculated by rating the difference between the percentage viability at the end and at the start of each period, divided by the length of the period. Linear interpolation was used to estimate density and viability of spores on days when no leaf samples were taken for the purpose of correlation with weather variables. To detect whether applications of *U. atrum* affected the level of strawberry leaf colonisation by *B. cinerea* and non-target fungi the data on potential leaf sporulation from the paired treatments were subjected to an analysis of variance.

RESULTS

Germination of *U. atrum* conidia in the suspensions used in the experiments varied between 89-100%. Samples of green strawberry leaves collected just after spraying gave similar results with an average of 84.7% germination of *U. atrum* conidia. Initial densities of *U. atrum* conidia on green strawberry leaves sprayed with a concentration of 2 x 10^6 conidia ml^{-1} averaged 3292 conidia cm^{-2} (Standard error of mean (SEM) = 174) and 2376 conidia cm^{-2} (SEM = 246) when leaves were collected after 1 hour and 24 hours, respectively. Densities of *U. atrum* conidia on unsprayed green strawberry leaves were on average 8.4 (SEM = 2.5) and 2.6 conidia cm^{-2} (SEM = 0.9), in experiment 3 and 4, respectively.

Survival of conidia

Densities of total and viable conidia of *U. atrum* decreased more quickly during the first week after spraying than in subsequent periods (exp. 1-2, Fig. 1; exp. 3-4, Fig. 2).

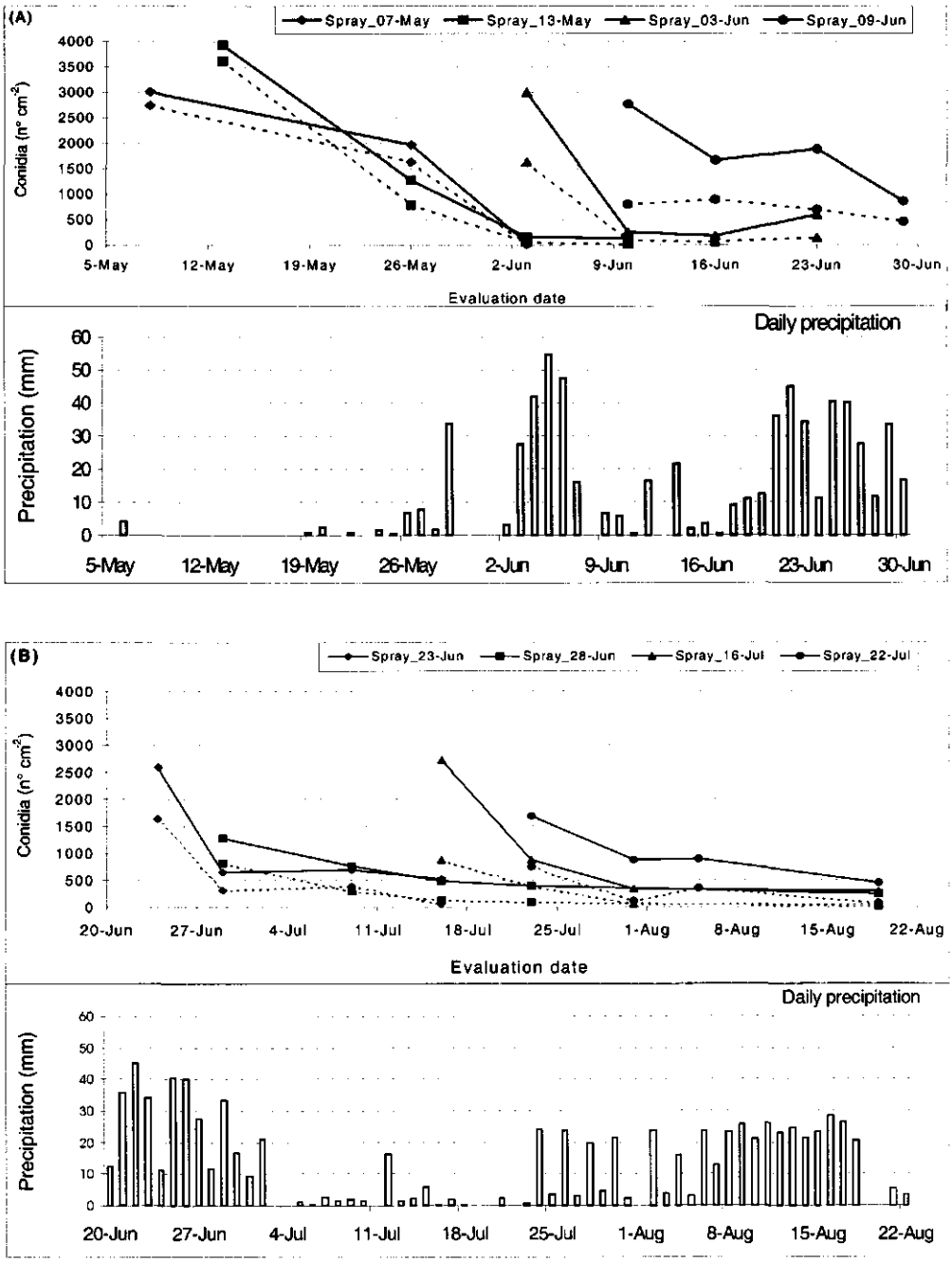

Figure 1. Total conidial density (solid line) and density of viable conidia (dotted line) after application of *U. atrum* on green strawberry leaves, and daily precipitation; (A) experiment 1, transplanting on 6 May 1998 and (B) experiment 2, transplanting on 19 June 1998.

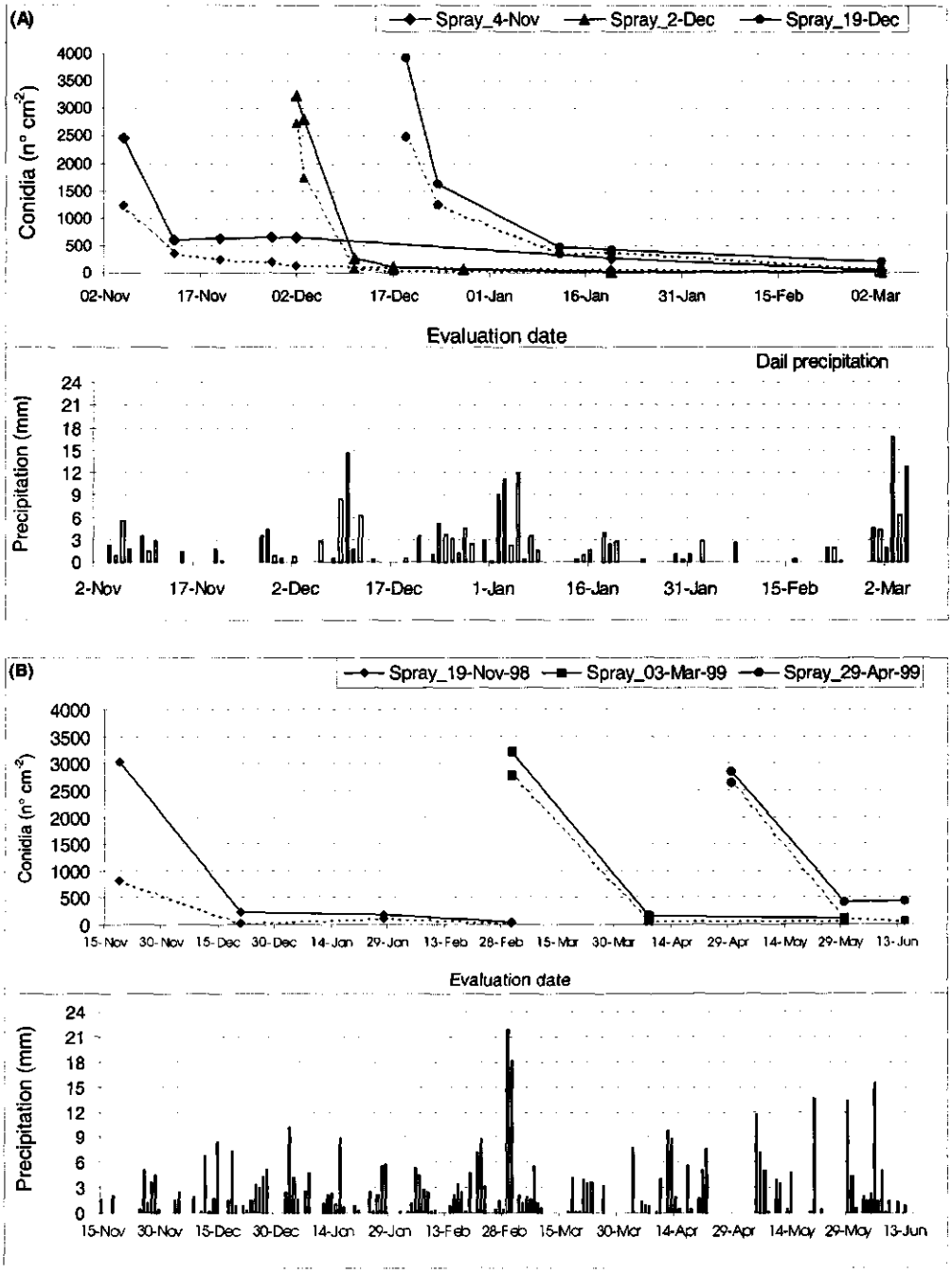

Figure 2. Total conidial density (solid line) and density of viable conidia (dotted line) after application of *U. atrum* on green strawberry leaves, and daily precipitation; (A) experiment 3, transplanting on 8 September 1997 and (B) experiment 4, transplanting on 21 August 1998.

The daily relative linear rate of decrease in total conidial density during the first seven days after spraying (pooled data of all sprays in experiments 1 and 2) was correlated with daily precipitation during the same period ($r = 0.70$; $P<0.05$). This correlation was not significant during the second week (8-14 days after spraying) and third week (16-20 days after spraying). When correlation's were calculated per spray, the relative rate of decrease in the density of *U. atrum* conidia between different evaluations was correlated with daily precipitation during the same interval only in the spray of 7 May 1998 ($r_s = 1.0$; $P<0.05$). No correlation was found between the relative reduction in rate of decrease in conidial density and other weather variables such as relative humidity and temperature. The density of *U. atrum* sprayed on any of the dates in 1997 and before 1 December 1998 (Fig. 2, A and B) was c. 80 conidia cm^{-2} (average of 4 sprays; SEM = 43) at the end of winter (3 March 1998 or 1999). After winter, 21% (SEM=7.6%) of the total leaf leaf disk area was colonised by *U. atrum* judging from sporulation after incubation. The decrease in spore density over time, relative to the first evaluation, was described with an exponential decay model. The model Y = exp (-0.10335* X) explained 16% of the variance, where Y is the normalised spore density and X is the number of days after spraying (up to 70 days) (Fig. 3). Regression analyse performed for each spray separately yielded significant fits to the exponential model in 11 of 14 sprays and the rate parameter varied between –0.2178 and –0.0464. The resulting models explained 80-99% of the variance. The exponential rate of decrease in spore density of these models was correlated with rain fall accumulation during the first seven days after spraying ($r = 0.55$, $P<0.05$).

The initial number of viable conidia was sometimes slightly and sometimes considerably less than the total number present, irrespective whether the first sampling took place after one or 24 hours (Fig. 1 and 2). The daily linear rate of decrease in the percentage of viable spores was correlated with the average temperature during periods of relative humidity higher that 95% ($r = 0.62$; $P<0.05$) in the first period of seven days after spraying, but not in the second (8-14 days) and third period (15-20 days) after spraying (exp. 1 and 2). Correlation's between rate of decrease of spore viability and other weather variables, such as relative humidity, radiation or precipitation, were not significant in any of the periods. The decrease in

spore viability pooled from all sprays could be described with the linear model Y=1+(-0.010 * X), r^2 = 0.11, where Y is the normalised spore viability, and X is the number of days after spraying (up to 70 days). When applied to the data from each spray separately, the linear model was significant in 4 of 14 sprays, with slopes ranging from –0.033 to –0.009.

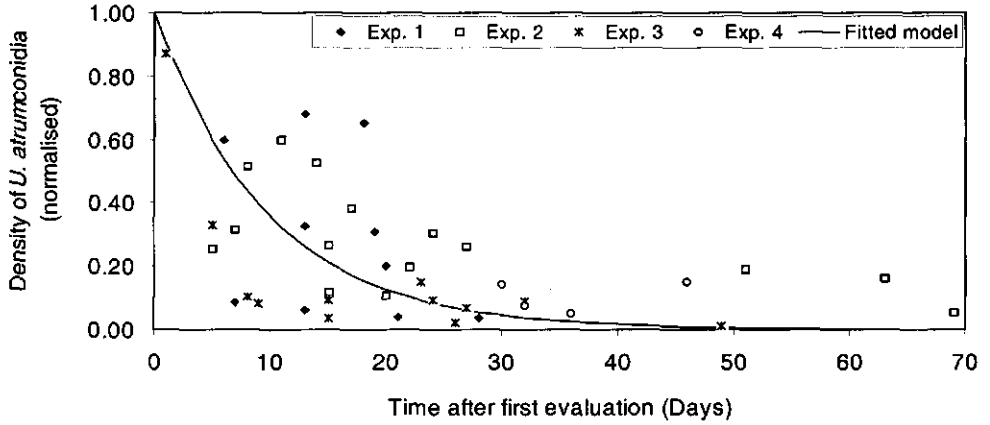

Figure 3. Density of *Ulocladium atrum* conidia on green strawberry leaves, normalised relative to the first count, data pooled from all sprays. The fitted model Y= exp(-0.10335*X) with df=40 has 16% of variance accounted for.

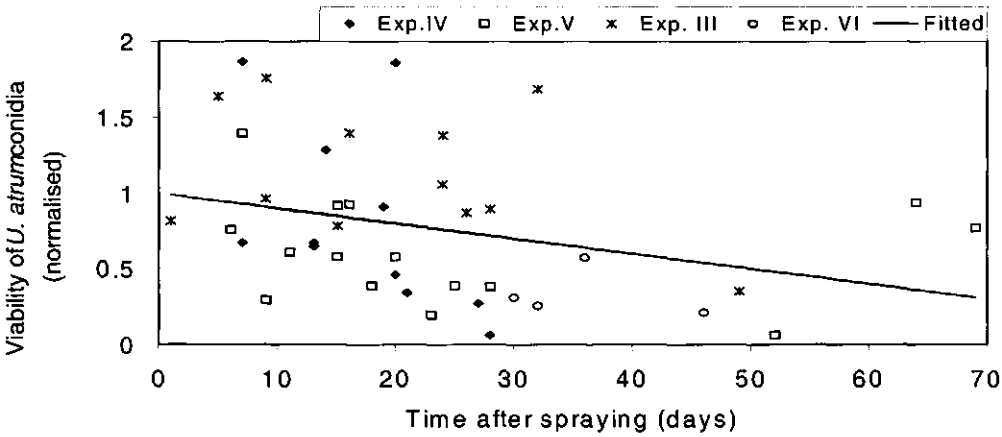

Figure 4. Viability of *U. atrum* conidia, relative to normalised viability in the of first assessment data pooled from all sprays. The fitted model is Y=1+(-0.010*X), r^2=0.11, df=41, P<0.01 (F-test).

Colonisation of *U. atrum*

The ability of *U. atrum* to colonise leaf tissue was measured by the percentage of leaf area with *U. atrum* sporulation after incubation under controlled conditions, defined as the potential sporulation of *U. atrum*. The potential sporulation of *U. atrum* on strawberry leaves after induction of necrosis showed a consistent decline over time after spraying (data not presented). Potential sporulation of *U. atrum* on this tissue increased with spore density, reaching maxima of c. 40, 30, 50 and 50% of the leaf area, for experiments 1, 2, 3 and 4, respectively (Fig. 5). Non-linear regression analysis was carried out with the logarithmic model $Y = b_0 + b_1 * \ln(X)$, where Y = potential sporulation area and X = spore density. The percentage of variance accounted for with this model was 23%, 26%, 55%, and 73% (F-test; $P < 0.05$) for the data from experiment 1, 2, 3 and 4, respectively (Fig. 5).

The relationship between the potential sporulation of *U. atrum* on naturally necrotic leaf tissue and spore density varied among experiments (exp. 1, 2, and 4) and neither linear and nor logarithmic models fit the data significantly in any case. However, the trend of *U. atrum* sporulation potential as a function of spore density on naturally necrotic tissue was similar to the trend on artificially induced necrotic tissue in experiments 2 and 4. In experiment 1, potential sporulation area of *U. atrum* on naturally necrotic tissue varied strongly independent of spore density.

Competition

Spore densities of *U. atrum* above 1500 conidia cm^{-2} resulted consistently in suppression of *B. cinerea* sporulation as compared to the unsprayed plot (Fig. 6). Linear regression analysis with spore density of *U. atrum* as independent variable and *B. cinerea* sporulation as dependent variable did not yield significant results (F-test, P=0.24). At densities lower than 1500 conidia cm^{-2} of *U. atrum*, suppression of *B. cinerea* sporulation on strawberry leaves after induction of necrosis was highly variable. At conidial densities higher than 1500, the presence of *U. atrum* significantly reduced the potential sporulation of *Alternaria* spp. in four of eight samples and to a lesser extent, in two of eight samples, of *Zythia fragariae* (Table 1). No effect on potential sporulation of *Cladosporium* spp. was detected.

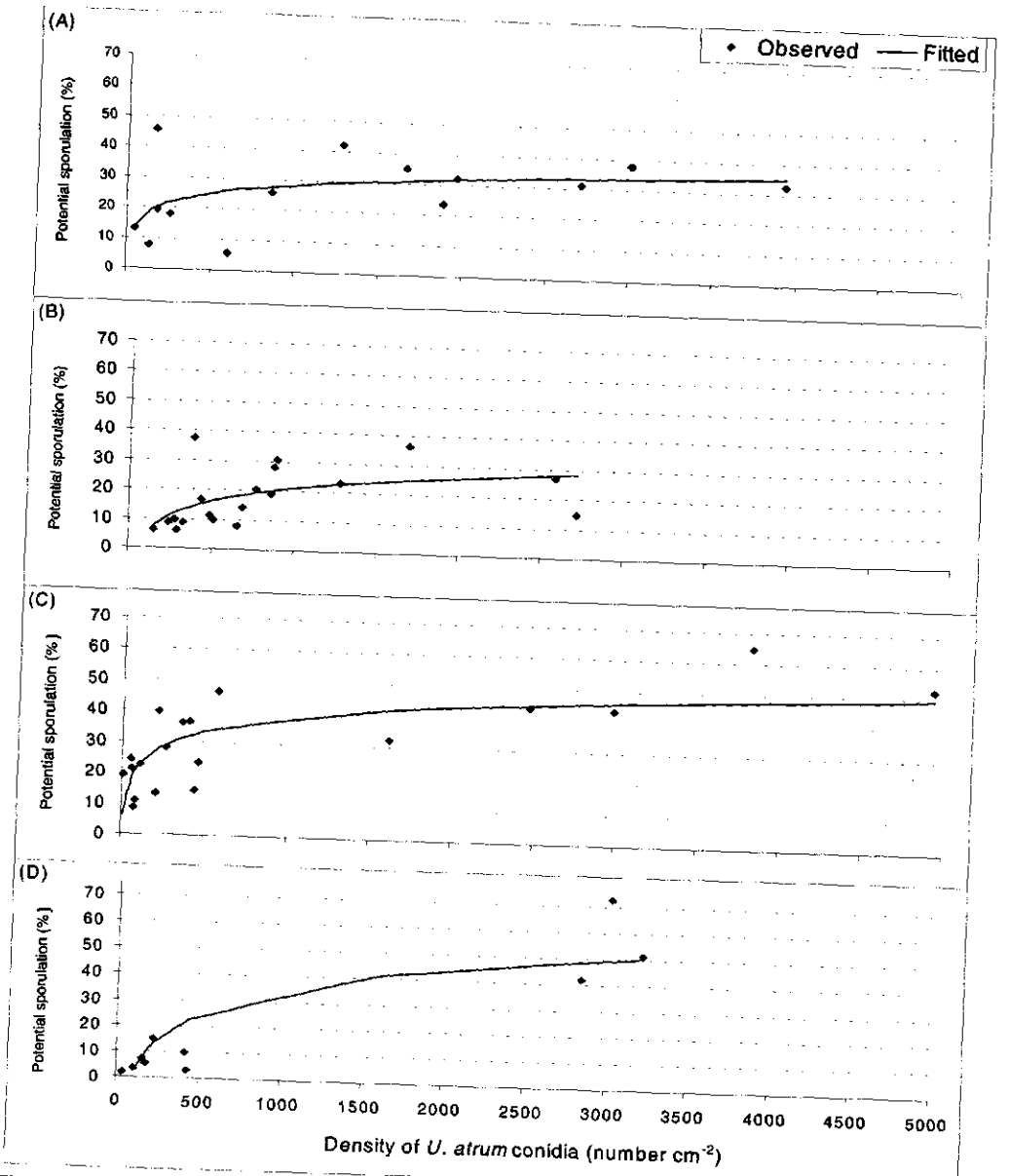

Figure 5. Potential sporulation of *U. atrum* as function of conidial density on artificially induced necrotic tissue of the green strawberry leaves. Data in experiments 1 (A), 2 (B), 3 (C) and 4 (D) originate from different sprays. Fitted values were calculated from equations (A)Y=-4.5+4.8*(Ln(X)), (B) Y=-28.4+7.2*(Ln(X)), (C) Y=-9.9+7.0(Ln(X)), (D) Y=-63.5+14.1(Ln(X)). Those non-linear regressions accounted for of 23, 27, 55, and 73% of the variance in experiments 1, 2, 3, and 4, respectively.

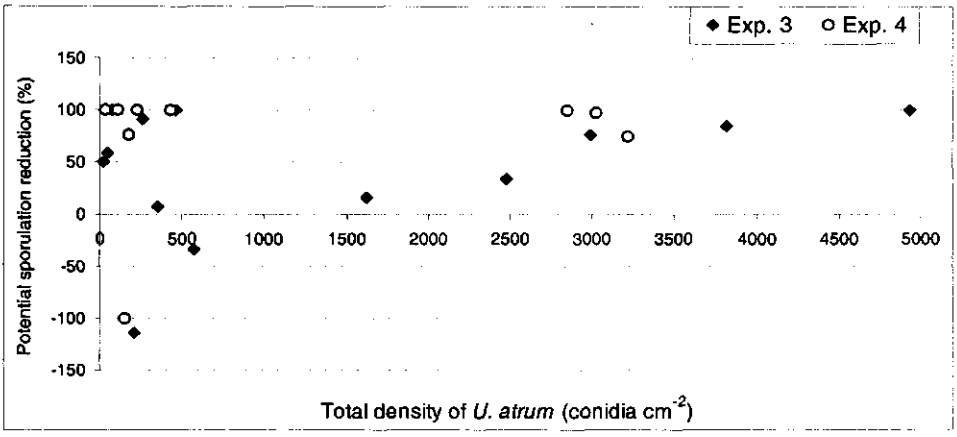

Figure 6. Suppression of *B. cinerea* potential sporulation by *U. atrum* as percentage of sporulation on unsprayed plants on artificially induced necrotic strawberry tissue as function of total conidium density in experiments 3 and 4. The graph shows selected data points that presented 1% or more potential sporulation of *B. cinerea* in the unsprayed sample.

The presence of *U. atrum* could affect potential sporulation of other unidentified filamentous fungi in only one of eight evaluations. At densities lower than 1500 conidia cm^{-2} of *U. atrum*, the potential sporulation of *Z. fragariae*, *Cladosporium* spp., *Alternaria* spp. and other fungi were sometimes lower and sometimes higher in *U. atrum* treated plots, but in most samples no significant differences between the *U. atrum* and control treatments were found.

Table 1. Potential sporulation area of *Zythia fragariae*, *Alternaria* spp., *Cladosporium* spp. and other fungi on artificially induced necrotic strawberry tissue from unsprayed and sprayed leaves, when total density of *U. atrum* conidia was higher than 1500 conidia cm^{-2} on sprayed leaves, experiments 3 and 4.

Application date of conidium suspension	Evaluation date	Total density of *U. atrum* conidia (number cm^{-2})	Potential sporulation area (%)[1]							
			Zythia fragarie		*Alternaria* spp.		*Cladosporium* spp.		Other fungi	
			Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed
Experiment 3										
Spray on 4-Nov-97	05-Nov-97	3818	0.3	0.0	0.3	0.8	0.1	0.1	1.3	2.0
Spray on 2-Dec-97	02-Dec-97	3228	21.0	20.0	11.3	5.8 *	5.0	1.8	6.5	3.1 **
	03-Dec-97	2808	25.8	20.5	10.7	7.0	3.1	2.0	13.1	8.0
Spray on 19-Dec-97	19-Dec-97	4935	13.3	16.1	11.2	4.2 **	4.7	2.3	6.9	4.7
	24-Dec-97	1620	15.6	5.9 *	10.8	7.2	8.3	4.3	6.1	3.9
Experiment 4										
Spray on 19-Nov-98	19-Nov-98	3022	10.3	11.7	14.3	3.7 *	0.0	1.0	10.7	12.0
Spray on 3-Mar-99	3-Mar-99	3222	22.0	16.0	0.3	0.0	3.0	0.0	9.0	6.4
Spray on 29-Mar-99	30-Apr-99	2850	28.7	5.0 *	6.7	0.7 *	1.0	0.0	21.7	15.0

[1] Values in the same row of the same fungus or group of fungi are significantly different compared in pairs (unsprayed and sprayed) by F-test (*0.01<P<0.05; ** (P<0.01). Values are averaged of 5 repetitions.

DISCUSSION

Persistence of *U. atrum* conidia

The initial density of *U. atrum* on strawberry leaves averaged 3292 conidia cm^{-2}, one hour after applying the antagonist at 2 x 10^6 conidia per ml. On lily leaves, Elmer and Köhl (1998) obtained with a similar concentration an average density of 4814 conidia cm^{-2}. However, the variance was considerably higher (SEM=1929) in comparison to what we found on strawberry leaves (SEM=174). The higher variability in lily can be attributed in part to the multi-layered structure of the canopy, which resulted in significant lower spore deposits on lower canopy levels. The density of *U. atrum* conidia on green strawberry leaves declined exponentially over time for most of the sprays (11 of 14). Elmer and Köhl (1998) found also an exponential decline of conidial density of *U. atrum* on lily leaves over the first 21 days after spraying. The exponential rate of decline in their experiments was c. -0.06 day^{-1} (recalculated from the presented data), which is close to the upper limit of the range we found in our study (-0.22 to -0.05 day^{-1}). Differences in rainfall are probably the major cause of the observed variability in the rate of decline. In our study, precipitation was the only weather variable correlated with the relative rate of decrease in conidial density of *U. atrum*, presumably by washing off the conidia.

Viability of *U. atrum* conidia, as assessed by vital staining, declined only slowly up to 70 days after application. For the pooled data, a linear decrease in conidial viability at a rate of c. 1% per day was found (Fig. 4). However, for 9 of the 14 individual sprays no significant linear decrease could be detected. The rate of decrease in conidial viability was correlated with average temperature during periods of high humidity (RH>95%) during the first week after application. As these conditions favour germination of *U. atrum* conidia, the reduction in the percentage of viable spores may be attributed to exhaustion of spores due to a high field germination rate (Köhl and Molhoek, in press). Elmer and Köhl (1998) found that conidial viability on lily leaves declined slightly till 7 days after field exposure, but then remained constant until at least 21 days after application. They measured conidial viability as potential germination, which gave probably an overestimation in later samples when a large proportion of the conidia had germinated in the field already. Yet, both their study in

lily and our study in strawberry indicate that loss of viability of *U. atrum* conidia under field conditions is quite low. Clearly, spore loss from leaf surfaces has a much stronger impact on the persistence of viable conidia of *U. atrum* than has loss of spore viability. This is in contrast with findings on the persistence of another biocontrol agent of strawberry grey mould, *G. roseum*. Yu and Sutton (1998) concluded from a study of the density dynamics of *G. roseum* applied in raspberry fields that rapid loss in germination ability was a key factor in low persistence. In their case, potential germination of *G. roseum* declined from over 80% just after application to less than 10% two days later.

Competitive colonisation of necrotic strawberry leaf tissue

Colonisation of necrotic strawberry leaf discs by *U. atrum*, measured by potential sporulation, increased with conidial density (Fig. 4), and at densities higher than 1500 conidia cm^{-2} leaf colonisation by naturally occurring *B. cinerea* was consistently reduced, often by more than 75% (Fig. 5). The inconsistent ability of *U. atrum* to reduce *B. cinerea* sporulation at lower conidial densities may be caused by the inability to out-compete existing colonisation by *B. cinerea* or other saprophytes (Kessel, 1999). At low densities, the coverage of the leaf surface by *U. atrum* conidia is also reduced, which increases the probability that naturally occurring *B. cinerea* escapes competition and colonises the vacant tissue. Moreover, these lower conidial densities were found mainly in the later samples, when the conidia were older and probably less vigorous.

Colonisation of naturally necrotic leaf tissue by *U. atrum* was generally somewhat lower and more variable than colonisation of leaf tissue with artificially induced necrosis, and suppression of *B. cinerea* sporulation at high conidial densities of the antagonist was less consistent. Sutton and Peng (1993) found that other antagonists of *B. cinerea* (*G. roseum, Trichoderma viride* and *Penicillium* sp.), were less effective in suppression of *B. cinerea* when applied to senescent strawberry leaves as compared to green leaves. *G. roseum* failed to suppress the pathogen when tested on leaves that had died naturally. They concluded that these antagonists are probably ineffective in tissues where *B. cinerea* was already present. The advantage of *B. cinerea* in competitive colonisation due to prior establishment is

probably also the explanation for our findings, but it should be noted that even in naturally necrotic tissues *U. atrum* tended to suppress *B. cinerea* sporulation considerably. On necrotic strawberry leaf tissue from the untreated control, potential sporulation of *U. atrum* was low (2% or less) and the fungal community was dominated by *Z. fragariae*, *Alternaria* spp, *B. cinerea*, and *Cladosporium* spp. (in that order). Application of large quantities of *U. atrum* conidia resulted in a partial displacement of these fungi (Fig. 5, Table 1). This effect on the naturally occurring fungal community is apparently temporary, as it disappeared some time after spraying when conidial densities of *U. atrum* fell below 1500 conidia cm^{-2}.

Implications for timing and formulation of *U. atrum* sprays

Given an initial density of c. 3000 conidia per cm^2 of *U. atrum* deposited on the leaves after application of a suspension of 2×10^6 conidia per ml at rate of 750 l ha^{-1}, the minimal effective density of 1500 conidia per cm^2 will be reached one week after application, according to the fitted model of exponential decline (Fig. 3). With this level of persistence effective suppression *of B. cinerea* on strawberry leaves can only be expected when *U. atrum* is applied less than a week before the leaves become necrotic. However, timing of sprays is complicated by the variability in patterns of strawberry leaf senescence and decomposition (Chapter 2), and a weekly application schedule would thus be required to suppress *B. cinerea* effectively. Improved persistence of *U. atrum* conidia on the leaf surface will reduce the need for frequent applications. Spore loss was the major factor affecting persistence, and was associated with precipitation. Therefore, research on improved persistence of *U. atrum* sprays should concentrate on formulations that enhance rain-fastness of deposited conidia.

Acknowledgements

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Chapter 5

ROLE OF PETALS IN GREY MOULD DEVELOPMENT OF STRAWBERRY CROPS

Abstract

Studies were conducted in annual crops of strawberry cv. Elsanta to assess the relative importance of petals as an inoculum source of grey mould in strawberry, and to identify during which period of flower and fruit development, the presence of petals has a significant effect on development of grey mould on fruits. In 1998, the incidence of *B. cinerea* on flower parts was assessed and the symptoms of grey mould on fruits were characterised with regards to their starting point. The incidence of *B. cinerea* on petals was 65-85% of those flowers that harboured *B. cinerea*. The starting point of symptoms was located underneath the sepals in 65-85% of fruits with grey mould, and petals were present at this site in about 50% of the cases. In 1999, four field experiments were conducted in which the effect of petal removal at different stages of flower and fruit development on incidence of grey mould was assessed. The incidence of grey mould on fruits with petals retained till harvest was circa 55% more than on fruits where petals were removed or had dropped naturally by the end of flowering, regardless of planting date or inoculum level of *B. cinerea*. The incidence of grey mould was hardly different between treatments where petals were removed at young flower stage, old flower stage, or green fruit stage. It is concluded that petals are an effective and stable source of inoculum for fruit infection by *B. cinerea*. Considering the high probability that retention of petals during fruit expansion and ripening results in fruit infection, the elimination of petals as an inoculum source appears worthwhile even when the incidence of petal retention on fruits is relatively low.

P. Boff, J. de Kraker, M. Gerlagh, and J. Köhl

INTRODUCTION

Botrytis cinerea has been reported as the main cause of grey mould of strawberry fruits since early in the twentieth century (Stevens, 1922). It has long been known that infection of strawberry fruit by *B. cinerea* does not take place directly through the outer skin of young fruit (Maas, 1984; Powelson, 1960). Green and white fruit buds are more resistant to *B. cinerea* than young and old flowers (Jarvis and Borecka, 1968), but ripe fruit are again more susceptible than white fruit (Pappas and Jordan, 1997). The resistance of the outer part of young strawberry fruit to *B. cinerea* is at least partially due to the presence of enzyme-inhibitory tannins (Jersch et al, 1989).

Observations on flower versus fruit infection led to the hypothesis that infection of strawberry fruit by *B. cinerea* may take place via infected necrotic flower parts adhering to the fruit surface or trapped between fruit and calyx (Wilkinson, 1954). *B. cinerea* spores need many hours of leaf wetness to complete the infection process (Maas, 1984). Flower parts that adhere to the fruit surface may allow water films to persist long enough for infection to be completed from spores trapped between these tissues (Jarvis, 1962a). After completion of the infection process, stem ends may become latently infected from colonised flower parts and the pathogen remains quiescent in the receptacle until ripening (Powelson, 1960; Jersch et al, 1989).

Different flower parts may serve to a greater or lesser degree as gateway for fruit infection (Pappas and Jordan, 1997). For example, stamens are more important than styles as sources of latent infection by *B. cinerea* both in growth chambers and under field conditions (Bristow et al, 1986). Colonised petals could also constitute a source of fruit infection. Incidence of grey mould on ripe fruit could even be predicted from the proportion of flowers with petal necrosis (Bulger et al, 1987). Boff et al (Chapter 2) also found a high correlation between petal area with sporulation by *B. cinerea* after incubation in moist chambers and post-harvest grey mould on fruits. However, the contribution of petals to fruit infection depends on their fate after fruit initiation. Petals start to detach soon after fertilisation of the pistils, unlike stamens and pistils that stay attached to the fruit. Nevertheless, some petals cling to the fruit and could contribute to fruit infection.

Most of the studies cited above were correlative in nature. However, the reports by Wilkinson (1954) and Jarvis (1962a) are rather anecdotal, and do not provide sufficient ground to determine whether control measures should be targeted at petals to eliminate this potential inoculum source, and if so, what the best timing of these control measures would be. It was therefore deemed to be important to re-examine the relative importance of petals as an inoculum source of grey mould in strawberry, and to identify during which period of flower and fruit development the presence of petals has a significant effect on development of grey mould. This research is part of a more general research programme on biological control of *B. cinerea* by *Ulocladium atrum* through competition in necrotic tissue (Chapters 3 and 6). The objectives of the research reported here were: (1) to document the distribution of *B. cinerea* among flower parts, (2) to describe symptom development on strawberry fruit in detail, and (3) to investigate the effects of petal removal at different stages of flower and fruit development on grey mould on ripe fruit.

MATERIALS AND METHODS

Experimental set-up

All experiments were performed using annual cropping systems of strawberries, cv. Elsanta, on a sandy soil at Wageningen, the Netherlands.

Objective 1 was accomplished by examining strawberry flowers in an experiment on effects of crop debris (dead leaves) removal on grey mould development. The experiment had two treatments (with and without sanitation, i.e. debris removal) in four randomised complete blocks. The experiment was carried out in 1998 and details are provided elsewhere (Chapters 1 and 2). Flower samples were collected on 21 July, 5 August, and 11 August 1998.

Detailed observations on symptom development on fruit (objective 2) were done in an untreated strawberry field in 1998, where no pesticide treatments were carried out. Healthy and diseased red fruit were harvested on 15, 20, 24, 27, and 30 August, 1998, and symptoms were examined by characterising the type and starting point on each fruit.

For objective 3, four additional experiments were carried out in small field plots of 84 strawberry plants, planted in three double-rows. In each experiment, treatments consisted on non-manipulated and manipulated flowers by removing petals at different flower and fruit development stages.

Incidence of *B cinerea* on flower parts (objective 1)

The incidence of *B. cinerea* on petals, stamens, and pistils was assessed in samples from strawberry fields of cv. Elsanta. Samples were taken from four untreated plots and from four plots where crop debris, except flower parts, were removed twice weekly. A sample of 20 to 30 flowers, each flower from a different arbitrarily chosen plant, was collected per plot on 21 Jul, 5 Aug, and 11 Aug 1998. Only flowers with petals and brown anthers were sampled. The flowers from each sample were put into plastic boxes (22-cm length x 14-cm width x 5-cm height) with wet filter paper at the bottom and incubated at 18 °C for 12 days, in the dark. Petals, stamens, and pistils were examined for the presence of sporulation of *B. cinerea* with a stereomicroscope, at 10-100 x magnification. Flower parts were considered colonised by *B. cinerea* when at least one conidiophore per conidium was present. Flowers were classified according to the presence of *B. cinerea* sporulation on the different flower parts. Seven classes were distinguished: flowers with presence of *B. cinerea* sporulation at the same time on petals, stamens and pistils (pet_sta_pis), on petals and stamens (pet_sta), only on petals (pet), only on stamens (sta), on stamens and pistils (sta_pis), on petals and pistils (pet_pis) or only on pistils (pis). The frequency of each class, per sampling time, was expressed as a percentage of the total number of flowers with *B. cinerea* sporulation.

Symptom initiation of grey mould on strawberry fruits (objective 2)

Symptom initiation of grey mould was checked on all fruits harvested from an untreated field of strawberry cv. Elsanta on 15 , 20 , 24 , 27, and 30 August of 1998. Fruits with grey mould were classified by characterising the type and starting point of symptoms as follows: (a) underneath sepals with petals present (undersep_withpet); (b) underneath sepals without petals present (undersep_nopet); (c) at the middle or tip of fruits (free_surf); (d) touching infected fruits (fruit_touch); (e) soil contamination

(soil_touch); (f) mummified fruits (mummy); (g) overall sporulation and unclear starting point (unclear); (h) physical surface damage (damage) and (i) pedicel grey mould (stem_rot). The frequency of each specific symptom was expressed as a percentage of the total number of fruits with grey mould, per harvest date.

Manipulative field experiments (objective 3)

Experimental design, treatments and crop management

Four experiments were carried out in small field plots of strawberry cv. Elsanta, the Netherlands in 1999. Cold-stored transplants kept at -2 °C until the day before planting, were transplanted on 6 May (exp. 1 and 2) and 19 June (exp. 3 and 4). Each experiment consisted of 84 plants in a field of 4.5 x 4.7 m, comprising three double-rows with three plants per meter row length. Spacing between double rows was 1 m and within double rows 0.5 m. Experiments 1 and 3 were conducted under natural inoculum and experiments 2 and 4 under enhanced inoculum pressure of *B. cinerea*. Enhanced inoculum pressure of *B. cinerea* was achieved by placing infected strawberries inside the plots from first white bud appearance till ripening. The infected fruits, about two per plant, were deposited in rows twice per week, in the middle of double rows and at the plot margins at 30 cm distance from the strawberry plants. The distance between the fruits at first introduction was 15 cm and additional fruits were placed in between the previous ones, without removing any old fruits. Experiments of natural and enhanced inoculum pressure of *B. cinerea* were separated by wide buffer strips of 100 m grass.

Treatments were as follows: petals were removed with the help of forceps at young flower (YouFlo), old flower (OldFlo) or at green fruit (GreFru) stage; petals had dropped naturally till old flower stage (FreeFall) or remained attached to the fruit till harvest (Harv); fruits from non-manipulated flowers not selected for presence or absence of petals (Control). All treatments were replicated three times, with the replicates grouped in double rows as blocks. Per treatment, 40-60 suitable flowers were arbitrarily chosen in each block and individually labelled by attaching a tiny label at the pedicel.

Mist irrigation was used to ensure water supply for plant development during dry periods. During flowering, irrigation was applied every second night from 21:00 till

6:00 hours (5 min per 90 min) to stimulate *B. cinerea* sporulation and infection. Pesticides were not sprayed and weeding was done manually.

Flower and fruit phenology

Labelled flowers of the first and second branches of four (exp. 1) and three (exp. 3) arbitrarily selected plants were monitored daily till fruit harvest. The number of petals per labelled flower from white bud appearance till harvesting was counted. These petals were either still attached to the receptacle or trapped between the sepals and the fruit. Development stages of flowers and fruits were classified as white_bud (top of petals visible), open flower (fully reflexed petals till petal fall), green fruit (seed formation), fruit expansion (white fruits till red colour appearance) and fruit ripening (red colour appearance till fully red fruit).

Petal colonisation

Removed petals, one per flower, of young flower (YouFlo), old flower (OldFlo) and green fruit (GreFru) treatments in all experiments were placed on water agar (1.5% of agar) in sterile plastic petri dishes (⌀14 cm) and incubated for 14 days, at 18 °C in the dark. The percentage area with sporulation of *B. cinerea* was estimated for each petal using a stereomicroscope at 20 x magnification. Data were averaged per treatment per block (n=3).

Grey mould

Ripe symptomless fruits and fruits with *B. cinerea* symptoms were picked twice per week. Healthy fruits were always picked separately from diseased fruits to avoid contamination during harvesting in view of post-harvest evaluations. Healthy and diseased fruits with non-specific symptoms were individually put into square plastic pots (5 cm high) and pots from the same block were placed side by side into plastic trays (50 cm length x 30 cm width x 7 cm height) with wet filter paper on the bottom. Each tray was sealed within a plastic bag and incubated 72 hours at 18 °C in the dark, to allow development of specific symptoms. After incubation, fruits were checked for the presence of *B. cinerea* sporulation.

Data analysis

Statistical analysis of grey mould incidence was performed by analysis of variance (ANOVA) followed by LSD-tests of angular-transformed values to separate treatment means (Snedecor and Cochran, 1989) using the computer Genstat 5 version 4.1 (Genstat Committee, Algorithm Group Inc.). The minimal level of significance was taken as P=0.05. The frequency of strawberry fruits over grey mould symptom categories and the frequency of *B. cinerea* over different flower parts were analysed using contingency tables and the Chi-square test ($P<0.05$). Pearson's (r) coefficient of correlation was calculated between the incidence of grey mould in the control and the potential sporulation area of *B. cinerea* on petals sampled at young flower, old flower, and green fruit treatment stages.

RESULTS

Incidence of *Botrytis cinerea* on flower parts

The frequency distribution of *B. cinerea* presence on the different senescent flower parts was slightly but significantly different ($\chi^2=12.8$, df=5) over different flower parts between untreated and sanitation plots, whereas the distribution of this frequency was not significantly different among sampling dates within treatments (Fig. 1). Petals and stamens alone or in combination, were responsible for 60-80% of *B. cinerea* incidence on flowers. Incidence of flowers with sporulation of *B. cinerea* on stamens alone or in combination with other flower parts (85-100%) was higher than on petals alone or in combination (65-85%), whereas this incidence on petals was higher than on pistils (20-40%). Of all possible combinations of flower parts infected, *B. cinerea* never occurred on the combination petal plus pistil, without occurring on stamens as well.

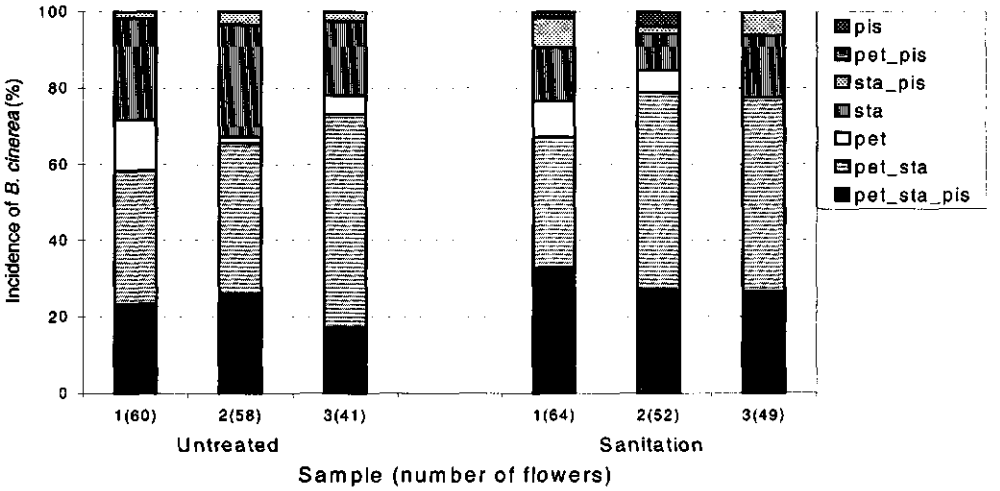

Figure 1. Incidence of *Botrytis cinerea* on senescent parts of strawberry flowers sampled on 21 Jul (1), 5 Aug (2) and 11 Aug (3) 1998 from untreated plots (Untreated) and plots where all crop debris were removed, except flower parts (Sanitation), Wageningen, the Netherlands. Each column shows the proportion of flowers on which *B. cinerea* was present at the same time on petals, stamens and pistils (pet_sta_pis), petals and stamens (pet_sta), only petals (pet), only stamens (sta), stamens and pistils (sta_pis), petals and pistils (pet_pis) or only on pistils (pis). Between brackets: number of flowers per sample.

Symptom initiation of grey mould on strawberry fruits

The distribution of strawberry fruits over grey mould symptom categories was significantly different among harvest dates (χ^2=148.6, df=32). The category 'touching infected fruits' and the category 'under sepal without the presence of petals' contributed most to this difference. The percentage of 'touching infected fruit' category was relatively high at first harvest, whereas the category 'under sepals without petals' was high at last harvest.

In 65-85% of the red strawberry fruits with grey mould, the starting point from where the symptoms had spread was located underneath the sepals (Fig. 2). About half of the fruits on which grey mould had started underneath the sepals had at least one petal trapped in between sepals and fruit. Petals were associated with 20-40% of all fruit rot. Grey mould rarely started at the middle or on the tip of the fruit in absence of visible damage (1.7-8.7%). Incidence of stem rot symptoms was low (4-6%) and these symptoms were only observed at the end of the harvesting period.

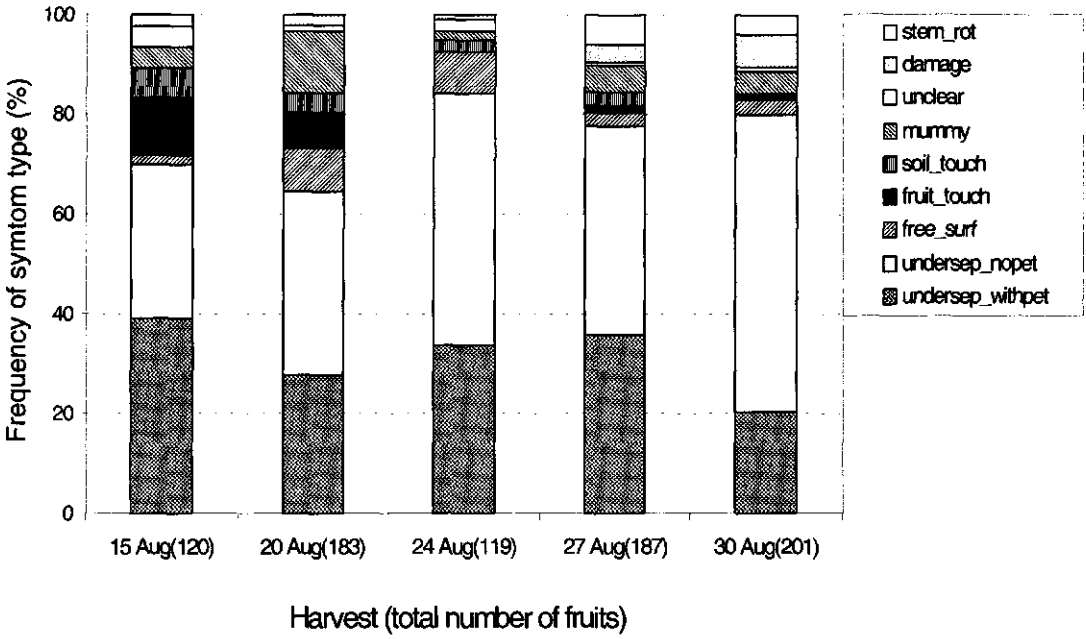

Figure 2. Frequency of type and starting point of grey mould from symptoms on strawberry fruits from untreated plants evaluated on 15, 20, 24, 27, and 30 August 1998. Symptoms were classified according to type and starting point as folow: under sepals with presence of petals (undersep_withpet) or without petals (undersep_nopet); at middle or tip of fruits (free_surf); touching infected fruits (*fruit_touch*); *soil contamination* (*soil_touch*); *mummified fruits* (*mummy*); *generalised* sporulation and starting position (unclear); surface physical damage (damage) or pedicel grey mould (stem_rot).

Petal manipulation experiments

Flower and fruit phenology

Petals dropped mostly during the open flower stages (Fig. 3). The retention of petals from the green fruit stage till harvesting was stable with on average of 0.3 petals per flower. The frequency of flowers with a minimum of one petal followed the same trend as the number of petals per flower. During flowering (about six days), 80% of all flowers dropped all petals and the remaining 20% retained at least one petal until harvest (20 days).

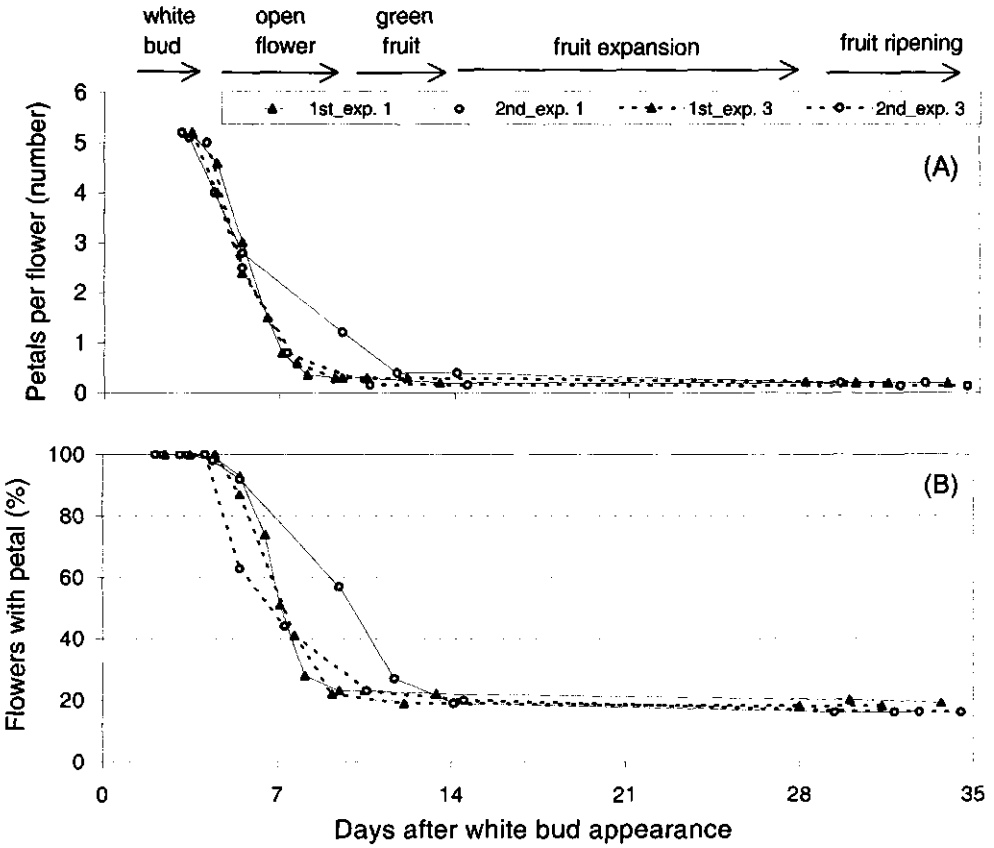

Figure 3. Petal retention of cv. Elsanta from white bud appearance till harvest, under field conditions, Wageningen, the Netherlands. (A) number of petals present per flower and (B) percentage of flowers with a minimum of one petal per flower. Data from first (1st_exp. 1, 69 flowers) or second (2nd_exp. 1, 38 flowers) branch of experiment 1, on 16 Apr 1999 and from first (1st_exp. 3, 51 flowers) or second (2nd_exp. 3, 43 flowers) branch of experiment 3, on 11 May 1999.

Potential sporulation of *B. cinerea* on petals

The potential sporulation area of *B. cinerea* was higher on petals from experiments 2 and 4 with an enhanced inoculum pressure than from experiments 1 and 3 with natural inoculum pressure (Fig. 4). In general, the potential sporulation area of *B. cinerea* increased with exposure time of petals to the inoculum present in the field. Petals sampled at green fruit stage always had higher *B. cinerea* colonisation than from young flower stages.

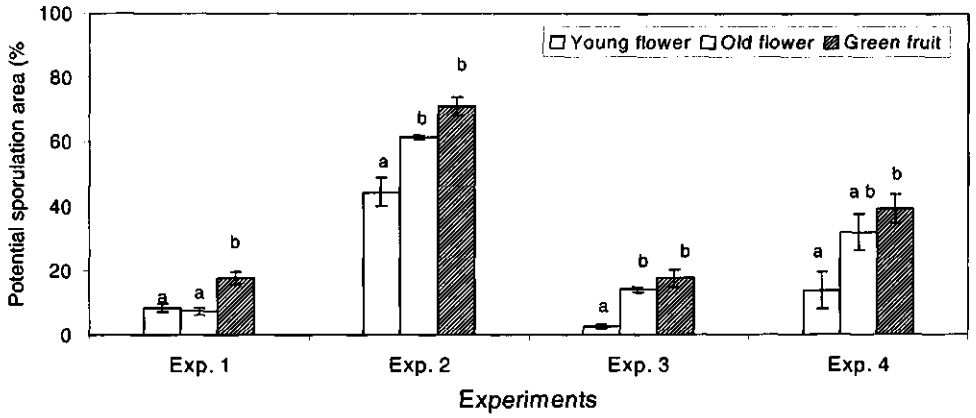

Figure 4. Potential sporulation area of *B. cinerea* on petals sampled from strawberry crops under natural inoculum pressure (exp. 1 and 3) and enhanced inoculum pressure (exp. 2 and 4) of the pathogen. Petals sampled at young flower, old flower or green fruit stage were incubated in moist chamber at 18 °C for 14 days, in the dark. Columns with the same letter in the same experiment are not significantly different (LSD-test; $P<0.05$). Bars are standard error of mean from three repetitions.

Grey mould

In all experiments fruits with grey mould were found, but the incidence was considerably higher in exp. 2 and 4 with the enhanced inoculum pressure (Fig. 5). Incidence of grey mould on fruits in the untreated control was 5-15% under natural inoculum pressure, and 25-55% under enhanced inoculum pressure of *B. cinerea*. Between experiments, the incidence of grey mould in the control was positively correlated ($df=2$, $P<0.05$) with the potential sporulation area of *B. cinerea* on petals sampled at young flower ($r=0.99$), old flower, and green fruit stage ($r=0.96$). When petals were present till harvest, significantly more grey mould occurred in comparison to all other treatments, irrespective the inoculum pressure of *B. cinerea* or the planting date (Fig. 5). For example, the difference in grey mould incidence between the treatments with petals present till harvest and with petals naturally dropped during flowering, was 51%, 51%, 54% and 66% in exp. 1, 2, 3, and 4, respectively. Incidence of grey mould on fruits with petals retained till harvest was higher under enhanced inoculum pressure (80-90%) than under natural inoculum pressure (55-60%) of *B. cinerea*. Removal of petals either at young open flower stage, old flower stage, or at green fruit stage had no or hardly any differential effect on grey mould of fruits. Among these three treatments, incidence of grey mould was highest in the

treatment where the petals had been removed at the green fruit stage in three of four experiments, but significant differences were only found in exp. 2 and 3. Incidence of grey mould was lowest on fruits that originated from flowers of which all petals had dropped naturally during flowering in three of four experiments (P=0.001), but did not differ significantly from incidence in the treatments where petals had been removed at the young or old flower stage, except in exp. 4.

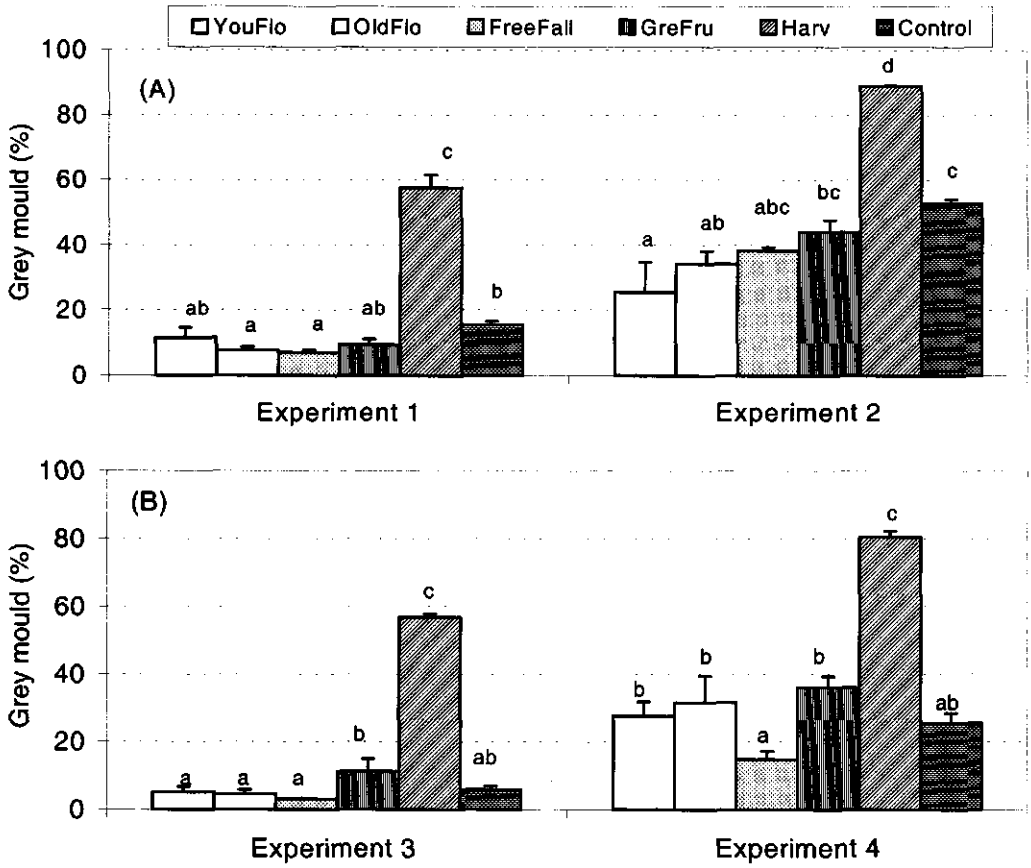

Figure 5. Grey mould of strawberry under natural (exp. 1 and 3) and enhanced (exp. 2 and 4) inoculum pressure of *B. cinerea*, in annual strawberry crops transplanted on 16 April 1999 (A) and on 11 May 1999 (B), Wageningen, the Netherlands. Treatments were: removal of petals at young open flower (YouFlo), old flower (OldFlo) or green fruit (GreFru) stage; petals fall naturally till old flower stage (FreeFall) or petals remain attached to the fruit till harvest (Harv); control fruits were from non-manipulated flowers (Control). Columns of the same experiment with the same letter are not significantly different (LSD-test; P<0.05).

DISCUSSION

Role of petals in strawberry grey mould

B. cinerea was found on the stamens, petals, and pistils of strawberry flowers (Fig. 1). The incidence on petals was somewhat lower than on stamens, but clearly higher than on pistils. Also considering the size of the different flower parts, there seems to be a differential susceptibility of these flower parts to colonisation by *B. cinerea*. Bristow et al (1986) found that *B. cinerea* readily colonises anthers and internal tissues of petals, whereas *B. cinerea* could also infect pistils, but hyphal growth was very slow and restricted.

The characterisation of the type and starting point of grey mould symptoms on strawberry fruits showed that in the majority of cases the infection had started underneath the sepals (65-85% of diseased fruits, Fig. 2). These results are very similar to those obtained by Powelson (1960), who found in a survey of five strawberry fields that 71-87% of the rotting fruits were infected at the calyx end, and suggested that the rot originated from infected stamens or sepals. However, our finding that petals were present in about 50% of the cases where grey mould symptoms had spread from underneath the sepals, indicates that also petals can play an important role in fruit infection. Since sepals hardly senesce till harvest and therefore *B. cinerea* cannot colonise the tissue into stem end or fruit receptacle, it seems that sepals play a minor role to establish infection during flowering or at early fruit development stages (Kamoen et al, 1985). The low incidence of grey mould symptoms starting at the middle or the tip of fruits, confirms previous observations that infections of intact fruit from airborne conidia rarely occur under field conditions (Jarvis, 1962a).

The experiments, in which the role of petals was investigated, demonstrated that the retention of petals till harvest greatly enhanced the incidence of grey mould (Fig. 5). The additional percentage of grey mould that can be attributed to the presence of petals from flowering till harvest was 51-65%, calculated as the difference in grey mould incidence between the treatment with petals present till harvest and the treatment in which petals had dropped naturally by the end of flowering. This percentage was remarkably constant across the experiments, which

represented different levels of inoculum pressure and, because of the two transplanting times, also different weather conditions. Apparently, petals adhering to the fruit surface are an effective and reliable source of fruit infection by *B. cinerea.* These petals may act as a saprophytic base for invading mycelium, or facilitate conidial infections by trapping spores and water in between the petal and the fruit surface. The latter mechanism is probably of minor importance, because the epidermis of strawberry fruit is not easily penetrated successfully by conidia of *B. cinerea*, unless the fruits are fully ripe (Jersch et al, 1989), whereas infection with mycelial plugs is more effective, even of half-ripe fruits (Jarvis and Borecka, 1968). Kamoen et al (1985), suggested that the saprophytic base provided by adhering senescent petals already detached at the base of the flower may function as protection for the pathogen against inhibitors from the plant, and allow the fungus to grow and produce toxins or enzymes needed to overcome plant resistance.

When does fruit infection through the presence of petals occur?

The presence of petals up to the green fruit stage did not or hardly influence the incidence of grey mould (Fig. 5). The period during which petals may act as a source of fruit infection by *B. cinerea* is apparently mainly between the green fruit stage and harvest. This can be explained with the results from Powelson (1960), who concluded that petals do not play a role in the establishment of latent infections in the receptacle during flowering, because an abscission layer causes the petals to fall before the fruit is invaded. Regarding mycelial or conidial infection of intact fruit, several studies have shown that intact young, green fruits are highly resistant to *B. cinerea*, whereas the susceptibility of the fruits increases towards maturity (Jarvis and Borecka,1968; Jersch et al, 1989). In addition to the greater susceptibility of ripening fruit, the effect of petal retention till harvest can be explained by the long exposure time of the petals to *Botrytis* inoculum resulting in higher levels of petal colonisation (Fig. 4). This long period of contact between the petals and fruits, enhance the probability of an infection event.

Implications for control

Whereas the retention of petals after the green fruit stage enhances the probability of fruit infection considerably, the need to eliminate this inoculum source will depend on how frequent petal retention is during fruit expansion and ripening. We observed that in strawberry cv. Elsanta at least one petal was retained on about 20% of all fruits (Fig. 3). This is relatively low and may explain why the role of petals has not been studied in detail, in contrast to the role of permanent flower parts (stamens, pistils, and sepals). The reduction in grey mould achieved by elimination of petals, as compared to non intervention (untreated) appears rather limited in our study. Only in the first two experiments significant differences were found between the incidence of grey mould in the control and in any of the treatments where petals had been removed or dropped naturally during flowering (Fig. 5). However, it can be argued that the observed level of reduction in grey mould incidence was in agreement with the expected level. Given that in the control treatment petals were present on 20% of the fruits, and that the presence of petals from flowering till harvest on all fruits enhances the incidence of grey mould by about 50%, an additional 10% grey mould would be expected in the control treatment as compared to treatments where petals were absent from the end of flowering onwards. The observed difference between these treatments and the control was on average 7.5%. Thus, considering the high probability that presence of a petal results in fruit infection, the elimination of petals as an inoculum source appears worthwhile even when the incidence of petal retention on fruit is relatively low.

Elimination of petals as an inoculum source of strawberry grey mould may be achieved by specific measures such as selection of cultivars that drop (almost) all petals or by physical removal of petals by blowing with compressed air. The latter approach has some success in viticulture, where it is used to remove flower caps from the grape clusters (Wolf et al 1997), but may be problematic in strawberry where the flowering period is long with overlapping generations of flowers. The conventional way to control grey mould is by application of fungicides during flowering (Maas, 1984). Spraying applications also result in blowing off petals, which may additionally contribute to grey mould control. Fungicides will reduce colonisation of petals most

effectively when applied protectively, i.e. before the arrival of inoculum of *B. cinerea*, because their post-infection effect on petals is poor (Kamoen and Jamart, 1975). Although the petals give rise to fruit infections much later during fruit development, the best timing of fungicide applications to control this source of inoculum will probably be during flowering. Colonisation of petals by *B. cinerea* may also be prevented or reduced by saprophytic competitors (Peng and Sutton, 1991; Köhl et al, 1995a). Providing the optimum time of application either by spraying fungicide or by introducing an antagonist for preventing *B. cinerea* colonisation on petals, it seems that the antagonist has higher potential to reduce fruit infection than fungicides. This can be argued because the antagonist, once it has colonised the petal tissues, can compete throughout fruit development, whereas the residual effect of a fungicide is always reduced in time. However, as the time advantage of one species over the other plays a crucial role in competitive colonisation of substrates by *B. cinerea* and the antagonist, large numbers of spores of the antagonist should be present on the petals as soon as these become accessible (Kessel, 1999).

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Chapter 6

BIOCONTROL OF GREY MOULD BY *ULOCLADIUM ATRUM* APPLIED AT DIFFERENT FLOWER AND FRUIT STAGES OF STRAWBERRY

Abstract

Grey mould of strawberries is an important disease resulting from flower and fruit infection by *Botrytis cinerea*. The saprophytic fungus *Ulocladium atrum* is a promising biological control agent for control of *B. cinerea* in strawberry and other crops. The objective of this research was to study the efficacy of *U. atrum* to control grey mould by a single application of a spore suspension (2×10^6 conidia ml^{-1}) at different flower and fruit development stages. Four experiments were carried out in 1999, two under natural and two under enhanced inoculum levels of *B. cinerea*. In each experiment, flowers and young fruits in six distinct stages of development were sprayed with either water or *U. atrum* suspension. *U. atrum* suppressed *B. cinerea* sporulation on petals by 15 to 54%. *U. atrum* was present on less than 30% of stamens and did not reduce the incidence of *B. cinerea* on these flower parts. The efficacy of the *U. atrum* sprays in controlling grey mould was low to moderate, and resulted on average in a reduction of 21% in disease incidence on ripe fruits. The moderate control efficacy is probably due to poor coverage of stamens with *U. atrum* spores, and a relatively low level of suppression of the colonisation of flower parts by *B. cinerea*. Significant reductions of grey mould in comparison to the control were found most frequently when the antagonist was introduced at late flowering or early fruit stages. Therefore, these are the most suitable stages to apply *U. atrum*. Further studies are needed to improve the spray coverage and persistence of *U. atrum* inoculum.

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INTRODUCTION

Grey mould, caused by *Botrytis cinerea,* is a major cause of fruit losses in strawberry crops (Hancock, 1999). Flower parts play an important role in fruit infection. Infection of stamens can result in the establishment of a latent infection in the receptacle (Powelson, 1960; Bristow et al 1986). The pathogen remains quiescent until ripening and may then cause fruit rot before or after harvest (Powelson, 1960; Jersch et al, 1989). Petals, adhering to the fruit surface and colonised by *B. cinerea,* may also constitute an important source of fruit infection (Wilkinson, 1954; Jarvis, 1962a). Direct infections of ripening fruit by conidia are of minor importance, and chemical control of grey mould was equally effective when fungicides were applied only during flowering as throughout flowering and fruit development (Jarvis, 1962a; Wilcox and Seem, 1994). Therefore, prevention of the establishment of *B. cinerea* in flowers with regular fungicide applications is nowadays the conventional method of grey mould control (Maas, 1984). However, increasing fungicide resistance of *B. cinerea* and public concern about the use of pesticides on food crops prompt the need for alternative control strategies (De Waard et al, 1993; Sutton, 1995; Ghini, 1996). Biological control by various filamentous fungi and yeasts is one of the methods that has shown good perspectives for non-chemical management of grey mould in strawberry (Sutton, 1995; Lima et al, 1997). Antagonists of *B. cinerea* are able to reduce the inoculum production of the pathogen during the flowering period, or may interfere with the infection process (Tronsmo and Dennis, 1977; Peng and Sutton, 1991). In perennial strawberry crops, crop debris is the major source of *B. cinerea* inoculum during flowering (Braun and Sutton, 1987), but in the annual cropping system as commonly encountered in the Netherlands, this source of inoculum is negligible (Chapter 2). Consequently, a biological control strategy aimed at suppression of sporulation of *B. cinerea* on crop debris will be less effective in annual strawberry crops. Given the role of flower parts in fruit infection by *B. cinerea,* antagonists may effectively control grey mould if they succeed to prevent or reduce pathogen colonisation of these flower parts.

The indigenous fungus *Ulocladium atrum* was found to exclude *B. cinerea* from necrotic plant tissue more effectively under field conditions than other

antagonists such as *Gliocladium catenulatum, Aureobasidium pullulans* or *Chaetomium globosum* (Köhl et al, 1995a). Laboratory studies showed that *U. atrum* is able to suppress tissue colonisation and sporulation by *B. cinerea* on necrotic leaves, stamens, and petals of strawberry (Köhl et al, unpublished). Under field conditions multiple applications of *U. atrum* crops resulted in significant reduction of pre- and post-harvest grey mould in annual strawberry crops (Chapter 3). An experiment conducted to optimise the application strategy of *U. atrum* during flowering indicated that the applied dosage could be lowered if the frequency of application was enhanced (Chapter 3). This may be due to an improved coverage of the flowers in a particular stage of development, because during the circa three weeks flowering period in strawberry, several overlapping generations of flowers are produced. When the effectiveness of *U. atrum* strongly depends on the development stage of the flower or young fruit at the time of application, application frequency should be high enough to reach all flowers in that particular stage. If the effectiveness of *U. atrum* is not strongly dependent on the development stage, timing and dosage should be such that as many flowers as possible are covered with an effective dose of *U. atrum.*

The objective of the present research was to study the effect of a single application of *U. atrum* on *B. cinerea* to flowers or young fruits at different development stages. The results can be used to further optimise timing and dosage of antagonist applications.

MATERIALS AND METHODS

Experimental design, treatments and crop management

Four field experiments were carried out with strawberry cv. Elsanta, on a sandy soil near Wageningen, the Netherlands. Cold-stored transplants kept at -2 °C until the day before planting were transplanted at 22 April (exp. 1 and 2) and 18 May 1999 (exp. 3 and 4). Each experiment consisted of 180 plants in a field of 4.5 x 10 m, comprising three double-rows with three plants per meter row length. Spacing between double rows was 1 m and within double rows 0.5 m. Experiments 1 and 3

were conducted under natural inoculum pressure and experiments 2 and 4 under enhanced inoculum pressure of *B. cinerea*. Enhanced inoculum pressure was achieved by placing infected strawberry fruits inside the plots, from first green bud appearance till ripening. Infected fruits were deposited twice per week at a rate of circa two fruits per plant between the double rows and outside the plot at 30 cm distance from the strawberry plants. The experiments with natural and enhanced inoculum pressure of *B. cinerea* were separated by a 100 m grass buffer.

Two treatments were applied: (1) spraying with *U. atrum* or not, and (2) timing of the spray application. For each of three timings (see bellow), paired plots were sprayed with either a *U. atrum* suspension (2 x 10^6 conidia ml^{-1} water with 0.01% Tween-80) or water with Tween-80. There were three plots for each treatment combination, and each plot had ten plants in double rows. *U. atrum* or Tween-water was applied once on all flowers branches per plot. During the spray application and till 5 min afterwards the plots were surrounded by a plastic sheet to avoid contamination of neighbouring plots. Prior to spraying, 20 to 30 flowers or fruits of the same development stage at the time of spraying were labelled per plot. Six development stages were distinguished: green bud, white bud, young open flower (yellow anthers), old flower (dark anthers at petal fall stage), green fruit and early white fruit.

U. atrum Inoculum production and field applications

Fresh conidial suspensions of *U. atrum* were prepared on each spraying day and kept at 4 °C till one hour before spraying. Conidia from a four week old culture of *U. atrum* grown on oat grains (Köhl et al, 1995a) were washed from the substrate using a camping washing machine (Nova, Belgium) filled with tap water containing 0.01% Tween-80. The resulting suspension was filtered through nylon gauze (200-μ mesh). The concentration was determined using a haemocytometer and adjusted to 2 x 10^6 conidia ml^{-1} before spraying. The dates of spraying were 5, 8 and 11 June 1999 for experiment 1 and 2, and 30 June, 1 July and 2 July 1999 for experiment 3 and 4. For each time of spraying, the viability of *U. atrum* conidia in the suspension was checked by spraying conidia on water agar and assessing conidial germination after 24 hours incubation at 18 °C in the dark. Suspensions were sprayed with an airbrush (Vega,

Thayere Chandler Inc, USA) at 100 kPa, held at 20 cm distance from the flower branches, at an application rate of 200 l ha^{-1}.

Microclimate

Weather variables were monitored in an extra strawberry plot at 50 m from the experimental plots, with identical irrigation and cultural management. Relative humidity and temperature were monitored with an electronic sensor (Pow 8-35 VDG, Rotronic AG, Bassersdorf, Switzerland) positioned at 30 cm height within the strawberry plot. Precipitation was measured by a recording rain gauge (Casella, London, UK). All data were stored each half-hour by a data-logger (Delta-T Devices Ltd, Cambridge, UK).

Spore load in the air

Spore load in the air was monitored using Rotorod samplers Mod. 20 (Sampling Technologies, Minnetonka, USA) with a non-retracting collector. Rotorods were positioned at 0.3 m height in the centre of natural and enhanced inoculum pressure fields, and 50 m outside the strawberry crop in the grass buffer. Runs were carried out on six days (30 May and 2, 8, 10, 15, and 16 June 1999) in experiments 1 and 2, and on seven days (18 , 23, and 25 June, and 04, 07, 15, and 22 July 1999), in experiments 3 and 4. Two runs per day, except on 18 June 1999 (one run) with 15 min duration per run were carried out between 9:00 and 12:00 hours, which is the most likely period for spore release of *B. cinerea* (Jarvis, 1980a). The spores of one rod per run were stained with cotton blue (2 ml lactic acid + 4 ml glycerol + 1.5 ml cotton blue at 1% + 2 ml demi water) and conidia of *B. cinerea* were counted on the 22 mm upper part of a rod using a microscope at 200 x magnification. The number of conidia of *B. cinerea* per cubic meter of air was calculated per run and averaged for two runs per sampling day (Sampling Technologies, Minnetonka, USA).

Fungal colonisation of flowers

Density of *U. atrum* conidia on petals

Density and percentage germination of *U. atrum* conidia on petals were assessed in experiments 3 and 4. In each plot, samples of ten young petals were arbitrary

collected from non-labelled flowers four hours after spraying and were incubated in plastic petri dishes with moist filter paper on the bottom for 15 hours at 18 °C in the dark to allow germination of *U. atrum* conidia. Subsequently, each petal was put on a glass slide with a drop of fluorochrome calcofluor white (Fluorescent brightener 28, Sigma; 0.02% w/v in 1M tris HCl buffer, pH 8.0). Germinated and non-germinated conidia within an arbitrarily chosen square of 1 mm^2 were counted using a fluorescence microscope (Zeiss Axioskop, filter 5, Germany). A conidium was considered germinated if at least one stained germ tube longer than half of a conidium diameter was observed. Density was estimated as the sum of germinated and non-germinated conidia.

Fungal colonisation of petals

Petals from unlabelled young flowers were collected four hours after spraying and when available three days after spraying. Ten to 15 petals per plot with a maximum of two petals from each flower, were placed on water agar (1.5% of agar in tap water) in sterile plastic petri dishes (Ø14 cm) and incubated for 14 days, at 18 °C in the dark. The percentage of petal area with *B. cinerea* sporulation was estimated using a stereomicroscope at 20 x magnification.

Fungal colonisation of stamens and sepals

Colonisation of stamens and sepals by *B. cinerea* and *U. atrum* was assessed on old unlabelled flowers (with dark anthers) collected one and four days after spraying of *U. atrum*. Ten to 15 flowers were sampled per spray. Flowers were placed into sterile petri dishes (Ø14 cm) on water/agar containing paraquat, at 10 mg l^{-1} (Gramoxone 200, Zeneca, UK), in such a way that the base of sepals touched the medium, in order to kill the sepals and allow both fungi to colonise the dead tissue. Petri dishes were incubated for 12 days at 18 °C in the dark. Stamens and sepals were examined for the presence of sporulation of *B. cinerea* and *U. atrum* with a stereomicroscope, at 10-100 x magnification.

Grey mould

All ripe symptomless fruits and fruits with *B. cinerea* symptoms were picked twice per week. Healthy fruits were always picked separately from diseased fruits to avoid contamination during harvesting in view of post-harvest evaluations. Healthy and non-sporulating fruits were individually put into square open plastic pots (5 cm high). Pots from the same block were placed side by side into plastic trays (50 cm length x 30 cm width x 7 cm height) with moist filter paper on the bottom. Each tray was sealed with a plastic bag and incubated for 72 hours at 18 °C in the dark to allow development of specific symptoms. After incubation, fruits were checked for the presence of *B. cinerea* sporulation.

Data analysis

The statistical analysis of data on grey mould incidence consisted of analysis of variance of treatments paired in blocks, using a computer package, Genstat 5 version 4.1 (Genstat Committee, Algorithm Group Inc.). The minimal level of significance was taken as $P<0.10$.

RESULTS

Density and survival *U. atrum*

The germination of *U. atrum* conidia from the applied suspensions on water agar was high and ranged between 89-95% for the different experiments. Samples of treated petals from young open flowers gave similar results with a conidial germination of 86% ± 5.3 (mean ± standard deviation). The initial density of *U. atrum* conidia on petals sprayed with a suspension at a concentration of 2×10^6 conidia ml^{-1}, and at a rate of 200 l ha^{-1}, was 5,655 ± 1621 conidia cm^{-2} (mean ± standard deviation) when petals were collected four hours after spraying. Conidia of *U. atrum* were not detected on petals collected from untreated young open flowers. No further evaluations on density and spore germination of *U. atrum* on petals could be done because they shrivelled.

Incidence of *U. atrum* on stamens sprayed at 2 x 10^6 conidia ml^{-1} suspension ranged from 20 to 37% when flowers were collected one day after spraying (Fig. 1). Four days after spraying, the incidence was 12 to 26%. Colonisation of *U. atrum* on sepals ranged from 60 to 78% per flower collected one day after spraying and from 45 to 80% for sepals collected four days after spraying.

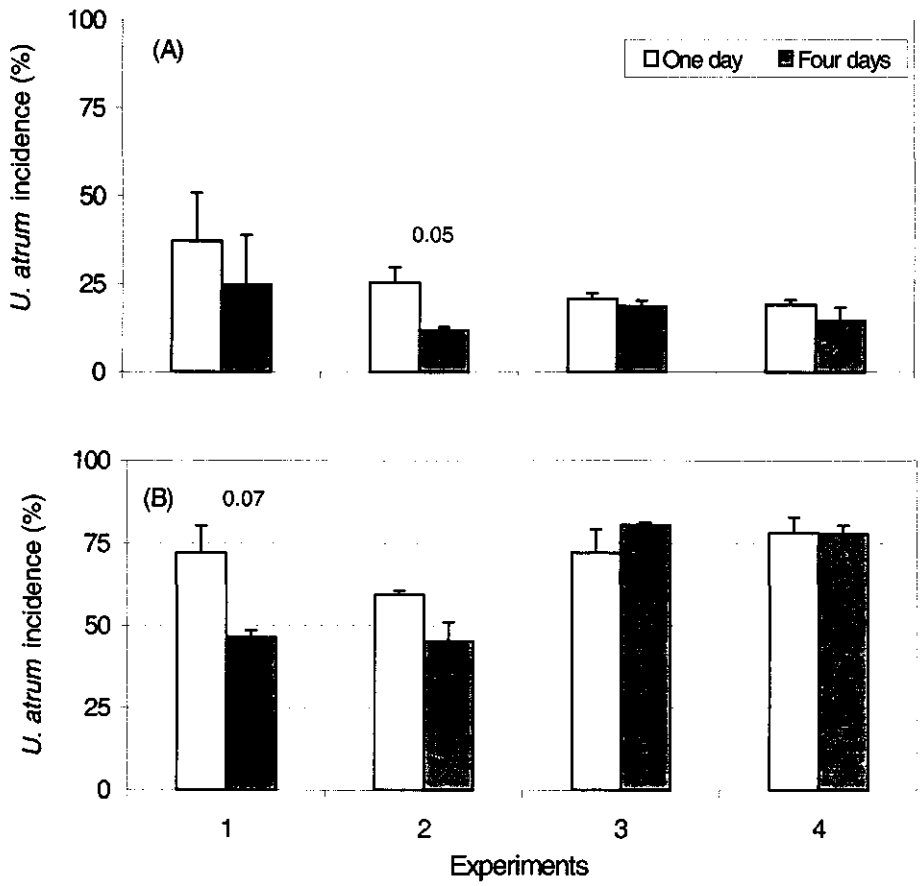

Figure 1. Incidence of *Ulocladium atrum* (mean ± S.E.M) on stamens (A) and sepals (B) of old flowers collected one day and four days after spraying the antagonist (2 x 10^6 conidia ml^{-1}) and incubated on paraquat/agar for 12 days, at 18 °C in the dark. Number at top of each pair of columns is the probability of F-test. Pairs without number at top have P>0.1.

Spore load and *B. cinerea* infection in control plots

The spore load in the air inside the fields with enhanced inoculum averaged 63.4 (exp. 2) and 175.2 (exp. 4) conidia per m^3 over the whole experiment, whereas the natural inoculum pressure inside the strawberry field was 10 and 14 conidia per m^3, respectively, in exp. 1 and 3. The average per run per day of the *B. cinerea* conidia load in the air was always higher in the enhanced inoculum fields compared either to the natural inoculum field or outside the fields. The *B. cinerea* inoculum load inside the fields with natural inoculum pressure was equal to the load outside the fields. The incidence of *B. cinerea* on flower parts of the control plots varied highly among the experiments and ranged from 34 to 95% on young petals and from 42 to 97% on old petals, which increased with experiments 1, 3, 2, and 4, in that order for both young and old petals (data not presented). Old flowers had a *B. cinerea* incidence ranging from 10 to 55% on stamens, and from 9 to 75% on sepals (Fig. 2). In the experiments with the enhanced inoculum pressure of *B. cinerea,* the sporulation incidence of the pathogen on sepals or stamens was always higher than in the experiments with natural inoculum pressure.

Suppression of *B. cinerea* colonisation of flower parts

U. atrum suppressed *B. cinerea* colonisation on young petals collected three hours after spraying of the antagonist significantly in two of four experiments (Fig. 3). On petals collected three days after spraying of the antagonist, a treatment effect on *B. cinerea* sporulation was found in one of two experiments. The suppression of sporulation of *B. cinerea* by *U. atrum* on petals, calculated as a percentage of the control, was negatively correlated with the level of *B. cinerea* sporulation on the untreated petals ($r=-0.89$, $df=4$, $P<0.05$). In fields with enhanced inoculum pressure of *B. cinerea*, the suppression of sporulation of the pathogen by *U. atrum* on petals was lower (15-37%) as compared to field with natural inoculum (38-54%).

Stamens sampled one day after treatment with *U. atrum* tended to have a lower incidence of *B. cinerea* in comparison to the control in one of four experiments ($P=0.08$) (Fig. 2). The incidence of *B. cinerea* on stamens collected four days after spraying of the antagonist was the same in both treatments.

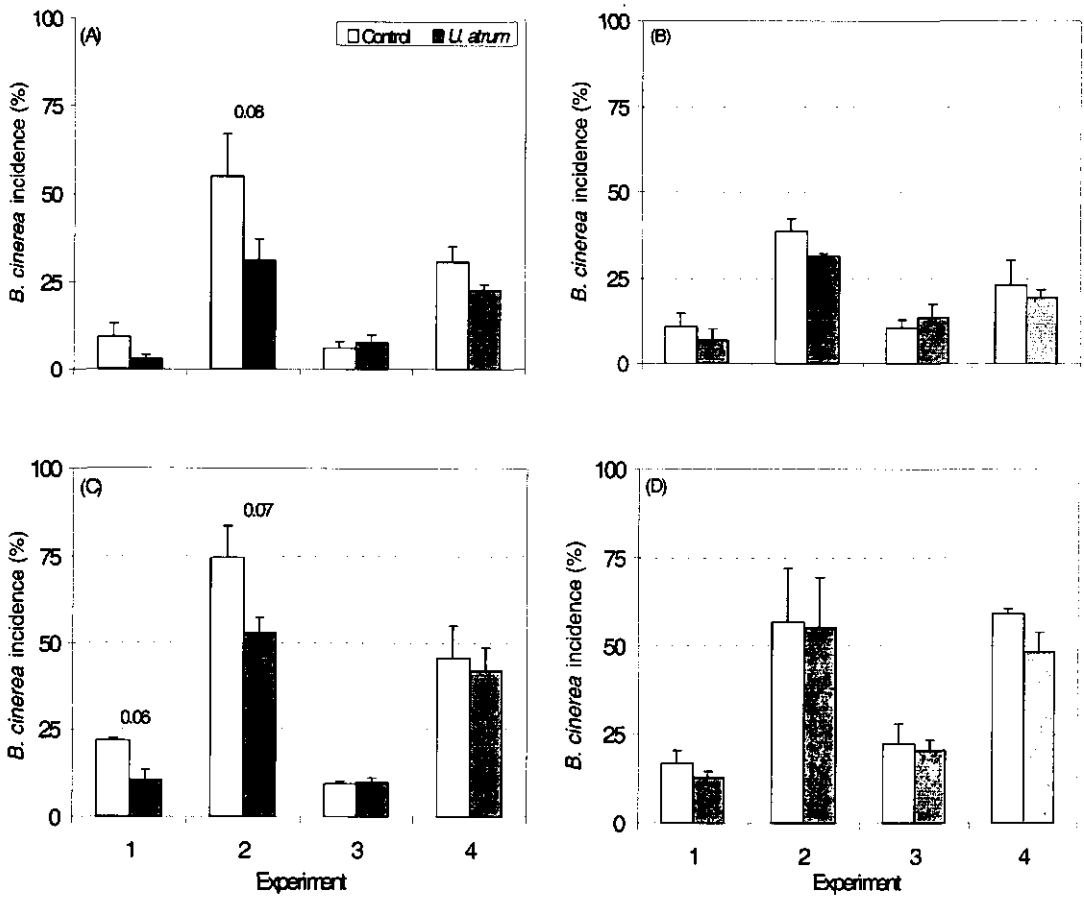

Figure 2. *Botrytis cinerea* incidence (mean ± S.E.M.) on stamens (A, B) and sepals (C, D) from old flowers of control and *Ulocladium atrum* treatments, collected one day (A, C) and four days (B, D) after spraying. Experiments 1 and 3 had natural *B. cinerea* inoculum; experiments 2 and 4 had enhanced *B. cinerea* inoculum. Number at top of each pair of columns is the probability of F-test. Pairs without number at top have P>0.1.

The incidence of *B. cinerea* on sepals sampled one day after spraying *U. atrum* tended to be lower than the control (P<0.07) in two of four experiments (Fig. 2). No difference of *B. cinerea* incidence between treatments was detected when flowers were collected four days after spraying the antagonist.

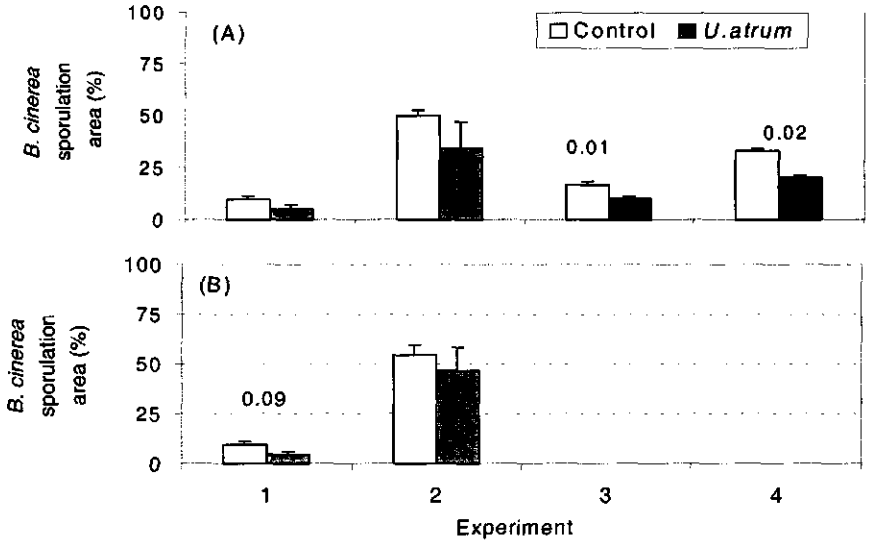

Figure 3. *Botrytis cinerea* sporulation on petals (mean ± S.E.M) from strawberry flowers of control and *Ulocladium atrum* treatments collected four hours (A) or three days (B) after spraying. Number at top of each pair of columns is the probability of F-test. Pair without number at top have P>0.1.

Grey mould reduction

The grey mould incidence of fruits in the control plot differed between experiments. High levels were found under enhanced inoculum pressure in experiment 2 (29-57%), whereas in the other three experiments (1, 3 and 4) the incidence ranged from 5 to 17% (Fig. 4).

The reduction of grey mould incidence by a single application of *U. atrum* (2 x 10^6 conidia ml^{-1}) was more effective when sprayed on old flowers or young fruits than on buds or young flowers. Spraying of *U. atrum* reduced grey mould when applied at the white bud stage in one of four experiments (P<0.02, exp. 4), at the old flower stage in two of four experiments (P<0.02, exp. 1; P<0.07, exp. 2), at the green fruit stage in two of four experiments (P<0.03, exp. 3 and 4), and at the white fruit stage in three of four experiments (P<0.01, exp. 3 and 4; P<0.1, exp. 2). No reduction of grey mould incidence was observed after a single application of the antagonist at green bud and young flower stages. For the eight cases with significant reduction of grey mould, a negative correlation was found between the level of reduction in the *U. atrum* treatment, calculated as a percentage of the control, and the incidence of grey mould in the control (r=-0.80, df=6, P<0.05).

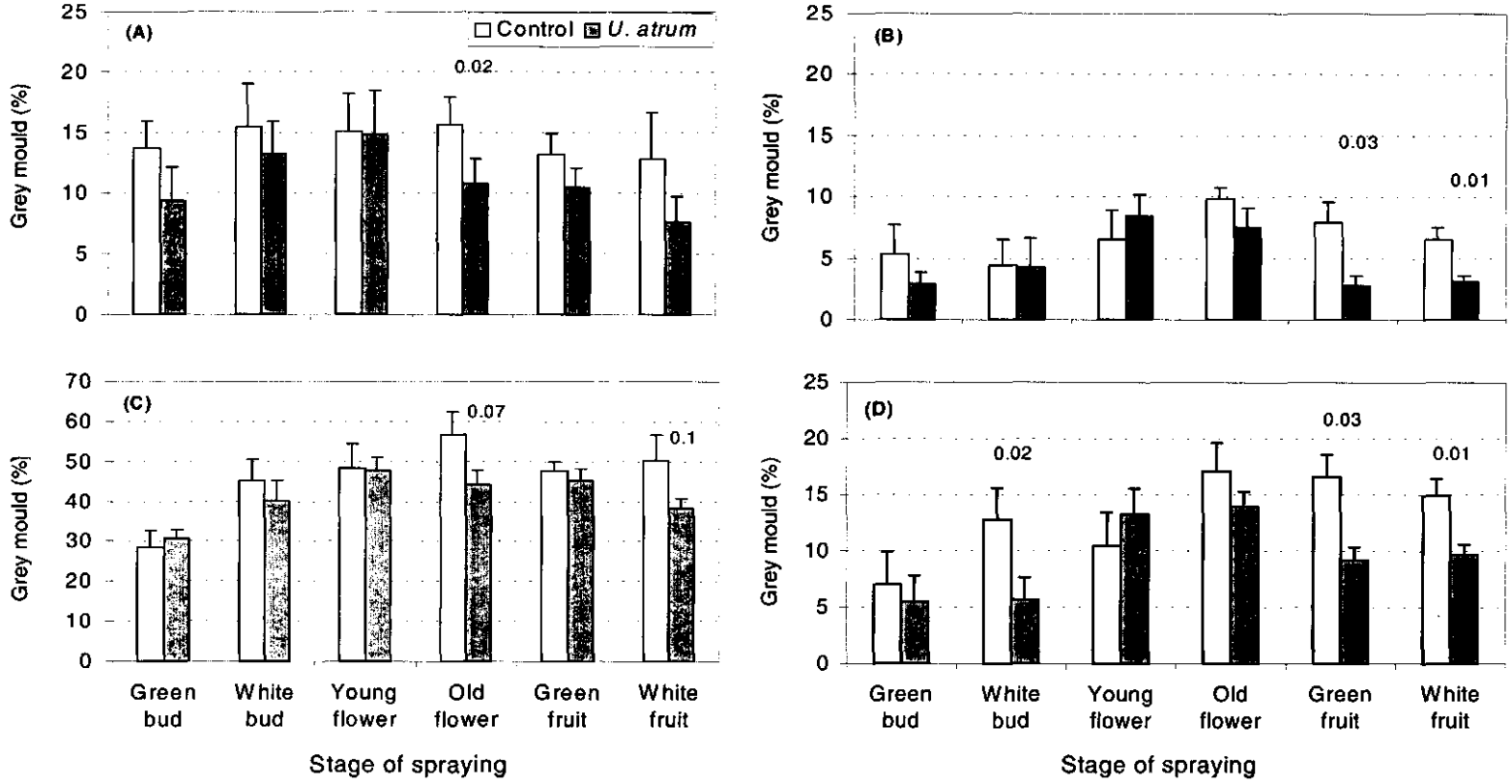

Figure 4. Grey mould incidence (mean ± S.E.M.) on strawberry fruits developed from flowers treated (*U. atrum*) or not (Control) with *Ulocladium atrum* at 2 x 10^6 conidia ml^{-1}, at different development stages. Natural inoculum pressure of *Botrytis cinerea*, transplanting on 22 April, exp. 1 (A) or on 18 May, exp. 3 (B) and enhanced inoculum pressure of the pathogen, transplanting on 22 April, exp. 2 (C) or on 18 May, exp. 4 (D), Wageningen, the Netherlands. Number at top of each pair of columns is the probability of F-test. Pairs without number at top have P>0.1.

DISCUSSION

Single applications of *U. atrum* to strawberry flowers and young fruits tended to reduce the incidence of grey mould on ripe fruits, on average by 21%. Statistically significant reductions (22-65%) were most frequently found when *U. atrum* was applied at the old flower, green fruit, or white fruit stage. The low to moderate control of grey mould can be explained by the poor coverage of flower parts with *U. atrum* conidia, in particular the stamens (Fig. 1), and the moderate suppression of the colonisation of flower parts by *B. cinerea* (Fig. 2 and 3). The observed levels of suppression of *B. cinerea* sporulation on petals (15-52%) were lower than expected, given the high densities of *U. atrum* conidia on sprayed petals (> 5,000 conidia/cm^2) and the high competitive ability of *U. atrum* on necrotic sterile petals in the laboratory (Köhl et al, unpublished). Experiments and model simulations on competitive colonisation of substrates by *U. atrum* and *B. cinerea* have shown that a lower initial spore density can be compensated by an earlier start (Kessel, 1999). Probably, *B. cinerea* has a time advantage on flower parts of strawberry, because the pathogen is able to infect petals, sepals, pistils and stamens before they senesce (Kamoen et al, 1985; Shiraze and Watanabe, 1985; Bristow et al, 1986; Bulger et al, 1987; Pappas and Jordan, 1997). *U. atrum* as a saprophyte can probably only colonise necrotic floral tissues. At higher levels of *B. cinerea* inoculum pressure, *U. atrum* sprays resulted in lower levels of reduction of petal colonisation by *B. cinerea* and of grey mould on fruits. The likely explanation is again the time advantage of *B. cinerea* in competitive substrate colonisation, with a higher probability of successful early infection of flower parts at a higher spore deposition rate. This time advantage is probably more important than the higher numbers of *B. cinerea* spores *per se*.

The best results with single sprays of *U. atrum* were obtained when the spray was applied at the old flower, green fruit, and white fruit stages, with significant grey mould reduction in seven of 12 cases (Fig. 4). In these stages of flower and fruit development, petals and stamens have started to senesce. The relative success of *U. atrum*, when applied at these stages, could be due to the high spore density present when the floral tissues become suitable for colonisation by the antagonist. *U. atrum* spores can remain viable on strawberry leaves in the field for several weeks

(Chapter 4). However, the number of spores decreased in time under field conditions due to losses during rainfall. On flower parts, such losses may occur during the period from spraying *U. atrum* at young development stages until the moment when necrotic tissue is available for colonisation by the antagonist. This may result in spore densities too low to compete successfully with *B. cinerea*.

Optimisation of the application programme of *U. atrum* to control grey mould differs from a fungicide spray schedule, because not only the relationship between flower or fruit development stage and susceptibility to *B. cinerea* infection has to be considered, but also the effect of development stage on colonisation by antagonist (e.g. Jarvis, 1969). Our results suggest that with the application technique and spore suspensions used in this study sprays can best be targeted at the old flowers and young fruit stages. This is convenient, as these stages are of relatively long duration as compared to, for example, the open flower stage. Multiple sprays at different stages of flower and fruit development may also have an additive effect.

The efficacy of single *U. atrum* sprays needs to be improved. This may be realised by enhancing the coverage of the flower parts, in particular the stamens, and by increasing the persistence spores on flower parts. This could be achieved by modifications in the application technique or by adding stickers to the spore suspension. Assuming that *B. cinerea* does have a time advantage in flower colonisation, especially when the inoculum pressure is high, *U. atrum* spores should be present in high numbers on flower parts as soon as these become available to the antagonist to minimise this time advantage. This could be achieved by targeting the earlier stages of flower development, but only when both the coverage and persistence of *U. atrum* sprays is high. Clearly, a differential ability of *B. cinerea* and *U. atrum* to colonise flower parts in subsequent stages of development will be crucial to the efficacy of *U. atrum* to control grey mould in strawberry.

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Chapter 7

GENERAL DISCUSSION

The epidemiological importance of components of the life cycle of *Botrytis cinerea* causing grey mould on strawberry crops depends on the crop production system, in particular if this is a perennial or annual system. Three years of field experiments with annual strawberry crops using waiting-bed transplants showed that grey mould is better correlated with weather conditions during pre-harvest and harvest than with incidence of *B. cinerea* on flowers (Chapter 2). However, when climatic conditions for symptom expression are suitable, the level of grey mould is determined by the level of *B. cinerea* colonisation on flower parts, as was demonstrated by the correlation between post-harvest grey mould and petal colonisation. The inoculum production of *B. cinerea* on crop debris did not contribute to spore load in the air during flowering, nor to the development of grey mould on fruits (Chapters 2 and 3). In perennial strawberry systems, it was found that the crop produces debris which *B. cinerea* can colonise and use for its reproduction. This provides the major source of inoculum for flower infection by the pathogen leading to grey mould development and fruit rot (Braun and Sutton, 1987). In the annual cropping systems we studied, the formation of debris is rather limited (Chapter 2). Consequently, inoculum of *B. cinerea* for flower infection and grey mould development on fruits was not determined primarily by the crop itself but by external sources (Chapters 3 and 5). Considering that the fungus can colonise and sporulate on a wide range of plant species, it appears that the availability of inoculum is unlikely to be a limiting factor in determining disease incidence in an annual cropping system of strawberry (Jarvis, 1980a; Berrie et al, 1998). Because locally produced conidia are not a major inoculum source, suppression of infection and colonisation on flowers and fruits is a more reliable strategy than suppression of sporulation on leaves (Chapter 2; De Kraker et al, in press). However, when rotten fruits were regularly placed in the crop, they enhanced inoculum load for flower infection increasing significantly grey mould incidence on

fruits (Chapters 5 and 6). Therefore, sanitation of rotten fruits infected by *B. cinerea* is a suitable strategy of grey mould control, whereas sanitation of dead leaves will not help to reduce disease incidence on fruits. Enhancing inoculum pressure of *B. cinerea* during flowering and fruit development did not always result in as high a grey mould incidence as expected (Chapter 5 and 6). A possible explanation is that weather conditions were not conducive to establishment of *B. cinerea* as latent infection in young fruits or to initiate direct infection through debris adhering to fruits (Powelson, 1960; Bulger et al, 1987).

Establishment of latent infection in the receptacle of young fruits as a consequence of colonisation of flower parts by *B. cinerea* is considered the major mechanism leading to grey mould disease on strawberry fruits (Powelson, 1960; Jarvis, 1962a). Flower parts, however, play different roles in the initiation of *B. cinerea* colonisation (Jarvis and Borecka, 1968; Bristow et al, 1986). We observed that petals significantly increased grey mould on fruits if they were present from green fruit stage onwards, irrespective of inoculum pressure of *B. cinerea* (Chapter 5). *B. cinerea* sporulation was observed on top of stamens, on anthers or at the end of the filament. Furthermore, most of the grey mould symptoms on fruits were initiated under the sepals. Half of such fruits had petals present till harvest, and grey mould symptoms started near these petals. (Chapter 4).

Spores freely deposited on the fruit surface are considered unimportant to start *B. cinerea* infection, but floral parts can serve as a bridge to facilitate penetration of the pathogen in fruit surfaces (Powelson, 1960; Jarvis, 1962b). This suggests that direct infection of *B. cinerea* on strawberry fruit surfaces is also an important mechanism to cause grey mould, and especially when debris is present during the development of fruits. In immature strawberry fruit tissue, antifungal proanthocyanidins are present preventing growth of the pathogen, so that *B. cinerea* remains quiescent (Jersch et al, 1989). Older cultivars of strawberry, such as 'Senga Sengana', show maturation from the inside to the outside of fruits, whereas in newer cultivars such as 'Elvira', genetically closely to 'Elsanta' used in our experiments, maturation progresses from the outer fruit tissue. Maturation patterns may affect the infection pathways of *B. cinerea* in the field. It is likely that cultivars with mature tissue first in the outside layers are more often directly infected. The role of petals in

this process is often underestimated because they form an abscission zone at their base and most of them detach not later than the old flower stage (Chapter 5). However, petals trapped between sepals and developing fruits, have a significant effect on grey mould development (Chapter 5)

In general, epidemiology of *B. cinerea* and grey mould development observed in our experiments (Chapters 2, 3 and 5) differed from those reported elsewhere (Jarvis, 1962a; Jordan, 1978; Braun and Sutton, 1987). This may be explained by differences in cropping systems (annual or perennial), but also effects of the cultivar, crop management, and local soil and weather conditions form possible explanations.

Biological control of grey mould on strawberry crops can be effectively achieved by using the antagonist *Ulocladium atrum*, in various spray programmes targeting the full canopy or the flower branches (Chapters 3 and 6). *U. atrum* is a strong competitor for necrotic substrate (Kessel, 1999). At the start of our study, suppression of inoculum production of *B. cinerea* on senescent and dead leaves was supposed to be the major mechanism to be exploited in grey mould control of strawberry (Köhl et al, 1995a). In other studies, using the same approach, *U. atrum* significantly controlled *B. cinerea* in greenhouse crops of pot roses (Köhl and Gerlagh, 1999), cyclamen (Köhl et al, 2000), and pelargonium (Gerlagh et al, unpublished). In our experiments, however, spray programmes of *U. atrum* starting at transplanting improved grey mould control only in one of five experiments, as compared to spraying during flowering only (Chapter 3). This suggests that either the antagonist is not a good competitor on the strawberry foliage before flowering or the foliage is not a proper niche where *U. atrum* could antagonise *B. cinerea*. Studies on the ecology of *U. atrum* in the phyllosphere showed that the antagonist is able to colonise the leaf tissue and suppress colonisation of naturally present *B. cinerea*, when the density of the antagonist is higher than 1500 conidia per cm^2 (Chapter 4). The same study showed that the initial density on leaves is about 3300 conidia cm^{-2}, when the antagonist is sprayed at a rate of 750 l ha^{-1} and a concentration of 2×10^6 conidia ml^{-1}. According to an exponential decay model fitted to the data, the density of the antagonist decreased in time and eight days after application the critical level of 1500 conidia cm^{-2} was reached. This is close to the weekly time interval that was used in the spray programme starting from transplanting (Chapter 3). It can be

concluded that the antagonist was introduced at a proper frequency for effective competition with *B. cinerea* on leaves. However, in a crop sanitation treatment, where a complete suppression of *B. cinerea* inoculum production on necrotic leaves was simulated by regularly removing of all senescent leaf tissues, no reduction of grey mould on fruits was found in comparison to the control (Chapter 3). This shows that excluding *B. cinerea* with *U. atrum* on the strawberry foliage of annual crops would not improve disease control, even if successful suppression of pathogen sporulation would take place (Chapter 4). Multiple applications of *U. atrum* show that spraying during flowering only can give a similar reduction of grey mould as spraying started from transplanting (Chapter 3). Thus prevention or reduction of infection during flowering is also involved in disease control.

Complementary studies with single applications of *U. atrum* targeted to different stages of flower and fruit development of strawberry, resulted in more frequent reductions of grey mould when the antagonist was introduced at late flowering or young fruit stages rather than at early flowering (Chapter 6). However, the effect of a single spray was not consistent for all experiments at the same stage of application of the antagonist. The variation could be due to poor distribution of the antagonist on stamens or lower suppression of *B. cinerea* on petals than expected. Multiple sprays focusing on the flowering period showed that the antagonist *U. atrum* could better control grey mould when the time interval was shorter (Chapter 3). When sprays were applied twice weekly, the amount of conidia per spray could even be reduced to 25% of the standard concentration without losing efficacy (Chapter 3). Flowers on strawberry crops in our experiments were produced each day and flower parts started to senesce after 3 to 5 days (Chapter 5). Considering the mode of action of *U. atrum*, which is by substrate competition, the antagonist is most effective when it can colonise the flower parts before or at the same time as the pathogen. This explains the need of frequent applications to reach all flowers at a proper time. Effective control of grey mould by *U. atrum* can then be achieved by blocking directly the flower and fruit infection. Since the applications of the antagonist in the field are done by spraying the whole crop, additional effects in disease control via reducing grey mould by suppression of *B. cinerea* sporulation on crop debris may also occur.

It was envisaged to discuss the outcomes of this study also under the conditions of Brazilian agriculture. In Brazil, strawberries are cultivated as annual crops lasting 80-140 days after transplanting in the production fields, depending on whether they are transplanted in the tropical or subtropical regions (Rebelo and Balardin, 1997; Lima, 1999). In Santa Catarina, as in other Brazilian states, strawberry crops are grown as part of small scale, mixed farming, family-run production systems (Verona et al, 1999; Schallenberger et al, 1999). The design of this agro-ecosystem is conducive to conversion into organic agriculture (Thurston, 1992; Pretty, 1995). When farmers adopt the principle of sustainable agriculture and fungicide use is reduced or eliminated, the development and use of biological control will become more important (Conway, 1996). In this scenario, the management of plant diseases as part of organic agriculture must consider internal resources and economic limitations (Verona, 1999; Tagliari, 1999). Augmentative biological control of plant disease based on mass-cultivation of indigenous antagonists can provide an effective tool during conversion or occasionally after that (Ehler, 1998; Bellows, 1999). Considering that the fungus *U. atrum* is naturally present in strawberry crops (Chapter 4), application of the antagonist, as referred to above and in the research chapters of this thesis, results in an augmentation of the resident population. Augmentation of naturally occurring antagonists, which are presumably well adapted and are part of the target system, enhances the probability that biological control of the pathogen will be successful. These antagonists also can be considered to have less environmental risks in comparison to newly introduced biocontrol agents (Waage, 1997; Conway, 1996). Given the differences in environmental conditions and crop management between the Netherlands and Santa Catarina State, BR, the use of a local isolate of *U. atrum* in Brazil to control grey mould on strawberry, would be promising during the conversion process if it would efficiently control the disease with a limited number of applications. Under the conditions of Santa Catarina State, *U. atrum* and other antagonists could best reduce grey mould by targeting flower and fruit infection, since the cropping system is annual and probably little debris is produced, similar to the annual crops using waiting-bed transplants in the Netherlands (Chapter 2). The rate of debris decomposition is high due to warm weather during spring till autumn and wet conditions during the whole year. In

addition to that, strawberry in Santa Catarina State is cultivated in small fields and crop rotation is frequently used (Verona, 1999). Thus, the sources of primary inoculum of *B. cinerea* are very diverse and aiming at suppression of inoculum production on debris may be inefficient. Sanitation by removing infected fruits with *B. cinerea* may have higher impact in Santa Catarina State than in the Netherlands because rain and high relative humidity are predominant, which provide conducive conditions for sporulation and infection almost every day.

In conclusion, it was demonstrated that the importance of epidemiological components of *B. cinerea* causing grey mould in annual strawberry crops is different from what was reported elsewhere for perennial cropping systems. In annual strawberry systems, little debris is produced and the inoculum formed inside the crop may not contribute substantially to the total inoculum that causes grey mould development. Therefore, control strategies of grey mould, such as biological control, in annual strawberry cropping systems using waiting-bed transplants should aim at protecting the flowers and developing fruits from saprophytic colonisation and infection by *B. cinerea.* The indigenous antagonist *U. atrum* can control grey mould on strawberry crops when sprayed during flowering as effective as sprayed from transplanting. Sanitation of crop debris will not help to reduce grey mould in cropping systems with low debris production, whereas sanitation of infected fruits can reduce the disease, especially under conducive weather conditions. Augmentative biocontrol of grey mould in strawberry crops by using an indigenous antagonist can meet local ecological conditions as is described above for Santa Catarina State, BR. However, reliable biocontrol strategies must consider a limited reliance on subsequent inundative releases.

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SUMMARY

Intensive crop production has led to various undesirable side-effects, including pesticide emissions to the environment. Conventional strawberry cultivation is a typical example of such intensive production with frequent pesticide applications, among others to control grey mould disease, caused by *Botrytis cinerea* Pers. (Teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel). The research described in this thesis deals with the development of an alternative management method of grey mould in annual strawberry crops. The main goal was to investigate biocontrol strategies using an antagonist that competes for substrate, *Ulocladium atrum.* This saprophyte occurs in the phyllosphere of several plant species in the Netherlands, and could be used as augmentative biocontrol agent in integrated and organic strawberry production systems. All experiments were carried out under field conditions in annual strawberry crops, cv. Elsanta, using waiting-bed transplants. In the first part (Chapters 2, 3, and 4) the potential for biological control of grey mould by *U. atrum* was assessed in relation to the epidemiology of *B. cinerea* and persistence of the antagonist under field conditions. Quantitative knowledge on disease epidemiology and the interaction with the antagonist are considered with special attention to annual cropping systems in comparison to perennial systems. In the second part, the role of flower parts for epidemic development is studied and the impact of targeting biocontrol at flower parts on infection of flowers and fruits by *B. cinerea* is investigated (Chapters 5 and 6). Finally, results of the research described in the thesis are discussed and prospects for application under Brazilian conditions, especially in Santa Catarina State, are addressed (Chapter 7).

Studies on the epidemiology of *B. cinerea* in five field experiments with annual strawberry showed that during the crop cycle, the availability of necrotic leaf substrate for spore production of the pathogen was generally low and varied between seasons and with the quality of transplants (Chapter 2). Inoculum production of *B. cinerea* inside the crop was not significant. The pathogen sporulated on a maximum of 15.5 cm^2 of leaf area per plant, after additional moist incubation, but sporulation in the field was hardly observed. *B. cinerea* incidence on flowers ranged from 5 to 96%, but no correlation was found with the potential spore

production on necrotic leaves nor with grey mould on fruits. Grey mould at harvest varied from 1.4 to 11.3% and was correlated with the average precipitation during the harvest period. The level of grey mould on strawberry fruits in the field depended more on conducive conditions for disease during fruit development and ripening than on the incidence of *B. cinerea* on flowers. However, when conditions for symptom expression were suitable, the level of grey mould was determined by the level of flower colonisation by *B. cinerea*, as was demonstrated by the correlation between petal colonisation and post-harvest grey mould.

The efficacy of the fungal antagonist *U. atrum* to control grey mould was investigated in eight field experiments with annual strawberry (Chapter 3). The antagonist was sprayed on the canopy, in different multiple application programmes and compared to control, fungicides and crop sanitation treatments. *U. atrum* significantly reduced grey mould at harvest in five of eight experiments. In two other experiments, incidence of grey mould was too low to evaluate the efficacy of the antagonist. Only in one of five experiments, sprays of *U. atrum* starting at transplanting resulted in better control of grey mould than sprays starting at the beginning of flowering. Crop sanitation did not affect the level of grey mould, which demonstrated that senescing or necrotic strawberry leaves were not a significant inoculum source of *B. cinerea*. Increasing the frequency of *U. atrum* sprays to twice per week gave better performance than weekly sprays when spore concentration was lowered from 2×10^6 to 0.5×10^6 conidia per ml. The results suggest that *U. atrum* can effectively reduce grey mould in annual strawberry crops by spraying during flowering.

In chapter 4, the ecology of *U. atrum* in the strawberry phyllosphere was studied to understand why the control of grey mould by the antagonist was sometimes erratic in field experiments. Strawberry leaves were sampled at various times after spraying *U. atrum* and checked for conidia persistence, potential sporulation of *U. atrum*, and suppression of colonisation of *B. cinerea* and other saprophytic fungi. Initial densities of *U. atrum* after spraying 750 l per ha of a suspension at 2×10^6 conidia ml^{-1} were more than 3000 conidia cm^{-2} of leaf. Densities declined more rapidly in the first week than in further periods. An exponential decay model for all experiments together account for 16% of the

variance, but similar models for separate sprays accounted for 80-99% of the variance. Rain was the main factor that reduced conidium density. The persistence of viable spores declined linearly over time at a rate of -0.01 till 70 days after spraying. The potential sporulation area of *U. atrum* as a function of spore density increased quickly till the density of 1500 conidia cm^{-2} was reached, resulting in a maximum of 50 % of the leaf area. Suppression of colonisation of naturally present *B. cinerea* was consistent only at densities higher than 1500 conidia cm^{-2} of *U. atrum*. At this level of conidium density, *U. atrum* could also compete successfully with *Alternaria* spp. but less efficiently with *Zythia fragariae* and *Cladosporium* spp. Further improvements on sporulation suppression of *B. cinerea* on leaf tissue by *U. atrum* should focus on rain fastness to increase the persistence of conidia.

Flowers are the key pathways for initiation of *B. cinerea* infection. The role of flower parts and in particular petals in the development of strawberry grey mould was investigated under two different levels of inoculum pressure of the pathogen (Chapter 5). Spatial distribution of *B. cinerea* sporulation on flower parts was assessed and symptom initiation of grey mould on strawberry fruits was characterised. Petals were removed at different flower or fruit stages. In flowers colonised by *B. cinerea*, the pathogen was found on petals in 65-85% of such flowers, whereas stamens were affected in 85-100% of the cases. Initiation of grey mould on fruits was localised under the sepals in 65-85% of diseased fruits and half of these fruits had petals still at the infection site. Incidence of grey mould on fruits with one or more petals still attached at harvest was 80-90% in fields where extra inoculum sources were added, and 55-60% in fields under natural inoculum pressure of *B. cinerea*. Removal of petals at flowering or during green fruit stage reduced grey mould incidence from 80-90 to 45% under enhanced and from 55-60 to 10% under natural inoculum pressure of the pathogen. Therefore, control strategies of strawberry grey mould, such as the use of antagonists, may successfully be implemented when excluding *B. cinerea* from such tissue, preventing latent or direct infection during the development of the fruits.

The efficacy of *U. atrum* to antagonise the infection of *B. cinerea* and to control grey mould was also studied by single applications at different flower or fruit development stages (Chapter 6). Two experiments were carried out under natural

and two under enhanced inoculum pressure of *B. cinerea*. In each experiment, six stages of flower-fruit development were distinguished and the two treatments, control and *U. atrum* application, were located in paired plots for each development stage. *U. atrum* suppressed *B. cinerea* sporulation on petals by 15 to 54%. *U. atrum* was present on less than 30% of stamens and did not reduce the incidence of *B. cinerea*. Significant reductions in grey mould in comparison to the control were found more frequently when the antagonist was introduced at late flowering or early fruit stages. Further studies are needed to optimise the distribution and timing of multiple sprays of the antagonist.

The importance of components of the epidemiology of *B. cinerea* causing grey mould on strawberry crops varies among production systems. In annual strawberry systems only a small amount of crop debris is produced and inoculum of *B. cinerea* produced within the field does not contribute significantly to the total spore load and to grey mould development. Therefore, in annual strawberry cropping systems using waiting-bed transplants, biological control strategies of grey mould should aim at the protection of flowers and developing fruits for saprophytic colonisation and infection by *B. cinerea*, and not target inoculum sources on leaf debris. The antagonist *U. atrum* can effectively control grey mould on strawberry crops when sprayed during flowering. Since the antagonist is sprayed on the canopy, additional effects on reducing grey mould by suppression of *B. cinerea* sporulation on crop debris may also occur but is considered to be less effective. Sanitation of crop debris will not help to reduce grey mould in production systems with little production of crop debris, whereas sanitation of infected fruits can reduce the disease especially under conducive weather conditions. Since *U. atrum* is considered an indigenous antagonist, application and introduction as described above can be replaced by the term augmentation. Augmentative biocontrol of grey mould in strawberry crops by using an indigenous antagonist could meet local ecological conditions as it is discussed for Santa Catarina State, BR.

SAMENVATTING

De intensivering van de landbouw heeft geleid tot verschillende ongewenste neveneffecten, onder andere emissie van bestrijdingsmiddelen in het milieu. Gangbare aardbeienproductie is een typisch voorbeeld van intensieve landbouw met herhaaldelijke bespuitingen, onder andere om de grauwe schimmel, *B. cinerea* te bestrijden.

Het in dit proefschrift beschreven onderzoek betreft het beheersen van dit pathogeen in de éénjarige teelt van aardbei uitgaande van wachtbedplanen. Hoofddoel was het verbeteren van strategieën van biologische bestrijding door gebruik van een antagonist die concurreert om substraat, *Ulocladium atrum*, en die van nature in de fyllosfeer van diverse plantensoorten in Nederland voorkomt. Biologische bestrijding van plantenziekten gebaseerd op vermeerdering door massakweek van inheemse antagonisten kan een effectief wapen verschaffen voor interventie in geïntegreerde in biologische aardbei productiesystemen, en tegelijkertijd het risico van ongewenste bijwerkingen vermijden dat vaak verbonden is aan import van antagonisten.

Alle proeven werden uitgevoerd met wachtbedplanten, cv. Elsanta, in éénjarige aardbei-teelt onder veldomstandigheden. In het eerste deel (Hoofdstukken 2, 3 en 4) worden studies aan epidemiologische aspecten van *B. cinerea* en effectiviteit van de antagonist *U. atrum* ter bestrijding van grauwe schimmel onder veldomstandigheden beschreven. Kengetallen van de epidemiologie van de ziekte en de interactie met de antagonist worden beschouwd, speciaal in relatie tot de vergelijking van éénjarige en meerjarige teeltsystemen. In het tweede deel van dit proefschrift wordt de rol van bloemdelen gedetailleerd bestudeerd en de interferentie van de biologische bestrijdingsstrategie met het infectieproces van *B. cinerea* op bloem en vrucht besproken (Hoofdstukken 5 en 6). Tenslotte worden de onderzoeksresultaten die in dit proefschrift beschreven staan en het perspectief van toepassing onder Braziliaanse omstandigheden, in het bijzonder in de Staat Santa Catarina, belicht (Hoofdstuk 7).

Studie van de epidemiologie van *B. cinerea* in vijf veldproeven met éénjarige aardbei toonde aan dat de beschikbaarheid van necrotisch bladweefsel voor

sporenproductie van het pathogeen in het algemeen laag was. Deze varieerde met de seizoenen en de kwaliteit van de wachtbedplanten (Hoofdstuk 2). De productie van inoculum van *B. cinerea* in het gewas was onbetekenend. Het pathogeen sporuleerde op maximaal 15,5 cm^2 blad per plant na vochtig incuberen in het laboratorium, terwijl nauwelijks sporulatie te velde werd waargenomen. De incidentie van *B. cinerea* op bloemen lag tussen 5 en 96%, maar een correlatie met de potentiële sporenproductie op necrotisch blad of met grauwe schimmel op de vruchten werd niet gevonden. Bij de oogst varieerde vruchtrot door grauwe schimmel tussen 1,4 en 11,3%, en was gecorreleerd met de gemiddelde neerslag tijdens de oogstperiode. Het niveau van grauwe schimmel op aardbeien in het veld hing meer af van gunstige condities voor de ziekte gedurende de ontwikkeling en rijping van de vruchten dan van *B. cinerea*-incidentie op de bloemen. Bij gunstige condities voor symptoom-expressie werd het niveau van aantasting van de vrucht door grauwe schimmel echter wèl bepaald door de mate van kolonisatie van de bloem door *B. cinerea*, zoals aangetoond door de correlatie tussen grauwe schimmel in de na-oogst fase en kolonisatie van de kroonbladeren.

De effectiviteit van de antagonistische schimmel *U. atrum* ter bestrijding van grauwe schimmel werd onderzocht in acht veldproeven met éénjarige aardbei (Hoofdstuk 3). De antagonist werd op het bladerdek gespoten in verschillende schema's met meerdere bespuitingen en vergeleken met een controle, fungicide toepassingen en sanitatie-behandelingen. Bij vijf van de acht proeven gaf *U. atrum* een significante vermindering van grauwe schimmel bij de oogst. In twee andere proeven was het optreden van grauwe schimmel bij de oogst zo beperkt, dat het effect van de antagonist niet te bepalen viel. Slechts in één op de vijf proeven resulteerden bespuitingen met *U. atrum*, die al bij het planten begonnen, in een betere bestrijding van grauwe schimmel dan wanneer bij begin bloei werd gestart. Sanitatie van het gewas had geen effect op aantasting door *B. cinerea*, wat aantoont dat verouderend of necrotisch aardbeiblad geen noemenswaardige bron van inoculum van *B. cinerea* was. Het opvoeren van de frequentie van bespuitingen met *U. atrum* van één tot twee keer per week leidde tot betere resultaten dan bij eens per week wanneer de sporenconcentratie werd verlaagd van $2x10^6$ tot $0,5x10^6$ conidiën

per ml. De resultaten geven aan dat *U. atrum*, gespoten tijdens de bloei, grauwe schimmel bij éénjarige aardbei-teelt effectief kan bestrijden.

In hoofdstuk 4 wordt de ecologie van *U. atrum* in de fyllosfeer van aardbei bestudeerd ter wille van het verklaren waarom de bestrijding van grauwe schimmel in het veld door de antagonist soms faalde. Aardbeiblad werd verzameld op verschillende tijden na bespuiting met *U. atrum* en gescoord op persistentie van de conidiën, potentiële sporulatie van *U. atrum*, en onderdrukking van sporulatie van *B. cinerea* en andere meest saprofytische schimmels. De dichtheid van *U. atrum* onmiddellijk na het spuiten van 750 l per ha van een suspensie van 2 x 10^6 conidia ml^{-1} lag boven 3000 conidiën cm^{-2} blad. Het aantal nam sneller af in de eerste week na het spuiten dan daarna, zodat een exponentieel model voor alle experimenten tezamen 16% van de variantie kon verklaren. Echter, exponentiële modellen voor de afname in sporendichtheden voor afzonderlijke bespuitingen konden 80-99% van de variantie verklaren. Regen was de belangrijkste factor voor de reductie van het aantal conidiën. De persistentie van kiemkrachtige sporen nam lineair met een factor -0,01 met de tijd af tot 70 dagen na bespuiten. Het potentieel sporulerend oppervlak bedekt met *U. atrum* als functie van de sporendichtheid nam snel toe tot een dichtheid van 1500 conidia cm^{-2}, waarbij het maximum van 50% van het bladoppervlak werd bereikt. Onderdrukking van kolonisatie door van nature aanwezige *B. cinerea* was slechts consistent bij dichtheden van meer dan 1500 conidiën cm^{-2} van *U. atrum*. Bij dit niveau van de conidiëndichtheid van *U. atrum* kon deze ook wedijveren met *Alternaria* spp., en, minder efficient, met *Zythia fragariae* en *Cladosporium* spp. Voor verdere verbetering van de sporulatie-onderdrukking van *B. cinerea* op bladweefsel door *U. atrum* moet vooral de regenbestendigheid van de conidiën van de laatste vergroot worden.

Bloemen zijn de sleutelroute voor de start van een *B. cinerea*-infectie. De rol van bloemdelen, en in het bijzonder kroonblaadjes, bij de ontwikkeling van grauwe schimmel van aardbei werd onderzocht bij twee verschillende niveaus van inoculum-druk van het pathogeen (Hoofdstuk 5). De ruimtelijke verdeling van sporulatie van *B. cinerea* op bloemdelen werd bepaald en het begin van aantastingssymptomen op de vrucht in kaart gebracht. Kroonblaadjes werden verwijderd bij verschillende stadia van bloei en vrucht-ontwikkeling. In 65-85% van door *B. cinerea* gekoloniseerde

bloemen herbergden de kroonblaadjes *B. cinerea* tegen 85-100% voor de meeldraden. Het begin van grauwe schimmel op de vrucht lag bij 65-85% van de zieke vruchten onder de kelkbladeren, terwijl in de helft van deze gevallen kroonblaadjes daar nog aanwezig bleken. Incidentie van grauwe schimmel op de vrucht bij nog aanwezig zijn van één of meer kroonbladeren tijdens de oogst, bedroeg 80-90% in veldjes met verhoogde inoculum-druk en 55-60% in veldjes met natuurlijke inoculum-druk van *B. cinerea*. Het verwijderen van kroonblaadjes bij de bloei of in het groene-vrucht-stadium reduceerde het optreden van grauwe schimmel van 80-90 tot 45% onder verhoogde inoculum-druk en van 55-60 tot 10% onder de natuurlijke druk van het pathogeen. Derhalve kunnen succesvolle bestrijdings strategieën van grauwe schimmel bij aardbei, zoals het gebruik van antagonisten, gebaseerd worden op uitsluiting van *B. cinerea* van de bloemdelen, zodat latente of directe infectie gedurende de ontwikkeling van de vruchten wordt voorkomen.

De effectiviteit van *U. atrum* om infectie door *B. cinerea* en grauwe schimmel te voorkomen werd ook bestudeerd door één enkele bespuiting toe te passen bij verschillende stadia van bloei en vrucht-ontwikkeling (Hoofdstuk 6). Twee proeven werden uitgevoerd onder natuurlijke en twee onder versterkte inoculum-druk van *B. cinerea*. In elke proef werden zes ontwikkelingsstadia van bloem tot vrucht onderscheiden, en de twee behandelingen, controle en toepassing van *U. atrum*, vonden plaats in gepaarde veldjes voor elk ontwikkelingsstadium. *U. atrum* gaf 15 tot 54% onderdrukking van sporulatie van *B. cinerea* op kroonblaadjes. *U. atrum* was aanwezig op minder dan 30% van de meeldraden, en gaf geen reductie van de incidentie van *B. cinerea*. Significante reductie van grauwe schimmel in vergelijking met de controle werd vaker gevonden bij toepassing van de antagonist laat in de bloei of vroeg in de vrucht-ontwikkeling. Verdere studie is nodig met meerdere bespuitingen om de toepassing van de antagonist te optimaliseren met betrekking tot verdeling en timing.

Het epidemiologisch belang van componenten van de levenscyclus van *B. cinerea* als veroorzaker van grauwe schimmel bij aardbei varieert van productie systeem tot productie systeem. In teeltsystemen waar het gewas weinig dood materiaal creëert draagt binnen het veld geproduceerd inoculum van *B. cinerea* niet of nauwelijks bij aan de sporenwolk, noch aan de ontwikkeling van grauwe

schimmel. Daarom dienen bestrijdingsstrategieën van grauwe schimmel, zoals b.v. biologische bestrijding, bij éénjarige teelt van aardbei op basis van wachtbedplanten zich te richten op de bescherming van bloemen en zich ontwikkelende vruchten tegen saprofytische kolonisatie en infectie door *B. cinerea*, onafhankelijk van de bron van het inoculum. De antagonist *U. atrum* kan grauwe schimmel op aardbei effectief bestrijden bij bespuiting tijdens de bloei. Aangezien de antagonist op het bladerdek gespoten wordt, kunnen ook additionele effecten op de reductie van grauwe schimmel door onderdrukking van sporulatie van *B. cinerea* op gewasresten optreden. Sanitatie van gewasresten zal in systemen met geringe productie van zulke resten niet helpen om grauwe schimmel te reduceren, terwijl verwijdering van geïnfecteerde vruchten, vooral bij voor de ziekte gunstig weer, de ziekte wel kan beperken. Daar *U. atrum* beschouwd wordt als een inheemse antagonist, kan toediening en introductie, zoals boven beschreven, vervangen worden door de term "vermeerdering". Biologische bestrijding van grauwe schimmel in aardbei door vermeerdering van een inheemse antagonist zou ook kunnen worden toegepast onder de ecologische condities die heersen in de Staat Santa Catarina, Brazilië.

RESUMO

O principal propósito desta tese foi de ensaiar uma estratégia de controle biológico de doenças de plantas, para que produtores pudessem ser orientados na conversão de sistemas agrícolas de alto uso de insumo para sistemas de agricultura orgânica e ecológica, socialmente adequada. O cenário de pesquisa valeu-se do manejo biológico do mofo-cinzento na cultura do morangueiro, causado pelo fungo *Botrytis cinerea* Pers., em experimentos conduzidos a campo, durante 4 anos, na Holanda.

O sistema de cultivo do morangueiro para produção de frutos varia de país para país dependendo do germoplasma utilizado, clima, perspectivas econômicas e preferência do próprio mercado. Cultivos a campo podem ser de ciclo anual ou perene. No sul do Brasil, por exemplo, as mudas são produzidas no período de outubro a março (primavera-verão), com transplante em março-junho (outono) e colheita a partir de setembro. Na Holanda, por comparação, o morangueiro é cultivado anualmente através do transplante de mudas em março-julho (primavera-verão) e produção em julho-setembro (verão). Neste sistema, utiliza-se mudas acondicionadas em câmaras frias a -2°C, que foram produzidas no verão-outono do ano anterior. O ciclo de cultivo no campo de produção varia entre 60 a 100 dias. Independentemente do país e sistema de produção utilizado, uma das principais doenças que pode atacar o morangueiro e causar danos econômicos é o mofo-cinzento. A doença se manifesta nos frutos e é decorrente da (1) infecção latente de *B. cinerea* estabelecida no receptáculo durante a floração, através da penetração do patógeno nas partes florais (sépalas e estamens) ou (2) infecção direta na superfície dos frutos durante o seu desenvolvimento, principalmente, via restos culturais aderidos na superfície dos mesmos. Baseando-se neste conhecimento, a maioria dos métodos de controle do mofo-cinzento tem sido desenvolvido através de um ou ambos dos seguintes princípios: a) redução na carga de inóculo do patógeno por supressão de esporulação, reduzindo a possibilidade de infeccão no período de floração e desenvolvimento de frutos; b) bloqueio ou redução da infecção das partes florais ou da superfície dos frutos.

A pesquisa a seguir descrita, desenvolvida nesta tese, teve o objetivo específico de estudar a epidemiologia do mofo-cinzento em morangueiro de cultivo anual e avaliar a eficácia de um antagonista nativo, o fungo *Ulocladium atrum*. Na primeira parte da tese (Capítulos 2, 3 e 4), foram estudados aspectos epidemiológicos de *B. cinerea*, persistência da densidade e viabilidade de conídios de *U. atrum* e a eficácia do antagonista no controle do mofo-cinzento. Na segunda parte da tese (Capítulos 5 e 6), foi estudado o papel das partes florais no desenvolvimento da doença e a eficácia do antagonista *U. atrum* com aplicações únicas nas flores. Finalmente, no Capítulo 7 são discutidos os resultados da pesquisa como um todo e também em perspectiva de seu potencial uso nas condições do estado de Santa Catarina, Brasil.

Estudos epidemiológicos de *B. cinerea*, em cultivo anual do morangueiro realizados em cinco experimentos, mostraram que a disponibilidade de restos culturais para a esporulação do patógeno esteve de modo geral baixa, mas variou conforme a estação de transplante e a qualidade das mudas utilizadas (Capítulo 2). A produção de inóculo de *B. cinerea* interna à lavoura foi desprezível. A incidência de flores com *B. cinerea* foi de 5 a 96 % e não correlacionou-se com o potencial de esporulação em folhas necróticas e nem com a incidência do mofo-cinzento em frutos. Incidência de mofo-cinzento em frutos durante a colheita foi de 1,4 a 11,3 % e foi correlacionada com a média diária de precipitação no período de colheita. O nível de mofo-cinzento em frutos de morangueiro esteve melhor relacionado com as condições climáticas durante o desenvolvimento e maturação dos frutos do que com a incidência de *B. cinerea* nas flores. Entretanto, sob condições favoráveis à expressão da doença, a colonização de *B. cinerea* em pétalas correlacionou-se com a incidência da doença em pós-colheita.

A eficácia do fungo antagonista *U. atrum* no controle do mofo-cinzento do morangueiro foi avaliada em oito experimentos a campo (Capítulo 3). O antagonista foi aplicado na parte aérea da cultura em diferentes intervalos e concentrações de conídios. Outros tratamentos incluídos foram: fungicida, testemunha e remoção de restos culturais. A remoção de restos culturais teve o objetivo de simular a total supressão da produção de inóculo de *B. cinerea* dentro da área de cultivo. *U. atrum* pôde reduzir a incidência do mofo-cinzento na colheita em cinco dos oito

experimentos conduzidos. Em apenas um experimento, aplicações do antagonista a partir do transplante de mudas resultou em menor incidência de mofo-cinzento do que aplicações a partir da floração. A remoção de restos culturais durante o ciclo da cultura não afetou o índice da doença na colheita. A redução da concentração da suspensão de inóculo de *U. atrum* de 2 x 10^6 para 0.5 x 10^6 conídios por ml, não afetou a eficácia do antagonista, irrespectivamente, à freqüência de pulverização.

No Capítulo 4, estudou-se a ecologia do antagonista *U. atrum* na filosfera do morangueiro. Folhas com uma única aplicação da suspensão conidial de *U. atrum* foram amostradas periodicamente e avaliadas quanto a persistência, viabilidade e competitividade do antagonista. Na parcela testemunha, o antagonista esteve presente na densidade de 2,6 a 8,4 conídios por cm^{-2}. Na parcela pulverizada, a densidade inicial alcançada pela aplicação de 750 l por ha da suspensão conidial de *U. atrum* na concentração de 2 x 10^6 conídios por ml foi superior a 3000 conídios por cm^2 de tecido foliar. A densidade declinou mais rapidamente nos primeiros oito dias do que nos períodos subseqüentes, de modo que o modelo de regressão exponencial pode explicar 16% da variância ocorrida. Chuva foi o principal fator relacionado com a redução da densidade de conídios do antagonista na superfície foliar. A persistência de conídios viáveis diminuiu linearmente à taxa diária de -0.01 até 70 dias após aplicação do antagonista. O potencial de área esporulante de *U. atrum* aumentou em função da densidade de conídios até o nível próximo a 1500 conídios por cm^2, resultando neste ponto uma área de esporulação de 50% da superfície foliar. A supressão da colonização do fungo *B. cinerea* foi apenas consistente em densidades superiores a 1500 conídios por cm^2 do antagonista.

O papel das partes senescentes das flores foi estudado com especial ênfase a pétalas e está descrito no Capítulo 5. Preliminarmente, foi avaliada a distribuição espacial de *B. cinerea* nas flores e a caracterização do sintoma inicial do mofo-cinzento nos frutos. Posteriormente, estudou-se o efeito da remoção de pétalas na incidência do mofo-cinzento nos frutos. Em pétalas, o patógeno esteve presente em 65 a 85% das flores. Estames foram colonizados em 85 a 100% dos casos. Sintomas iniciais do mofo-cinzento estiveram localizados sob as sépalas em 65 a 85 % dos casos, dos quais metade apresentaram presença de pétalas. Incidência do mofo-cinzento em frutos com uma ou mais pétalas presentes foi de 80 a 90 % em

parcelas sob alta densidade de inóculo e de 55 a 60 % em parcela sob condições normais de pressão de inóculo. A remoção de pétalas até o estágio de frutos verdes em comparação com sua retenção até a colheita reduziu a incidência do mofo-cinzento de 80-90 para 45%, e de 55-60 para 10%, sob condições de alta pressão ou de pressão normal de inóculo de *B. cinerea*, respectivamente. Estratégias de controle do mofo-cinzento do morangueiro, como o controle biológico, poderia melhor ser implementados pela exclusão da colonização do patógeno neste tecido floral.

A eficácia do uso de *U. atrum* no controle do mofo-cinzento foi, também estudada com uma única aplicação do antagonista em diferentes estágios de desenvolvimento da flor ou fruto do morangueiro (Capítulo 6). Dois experimentos foram conduzidos sob pressão natural e dois sob alta pressão de inóculo de *B. cinerea*. Em cada experimento, seis diferentes estágios de flor ou fruto foram identificados e etiquetados apropriadamente em parcelas pulverizadas e não pulverizadas pelo antagonista *U. atrum*, dispostas em par. *U. atrum* pode suprimir a esporulação de *B. cinerea* de 15 a 54% da área de pétalas. *U. atrum* esteve presente em menos de 30% dos estames de cada flor e não reduziu a incidência do patógeno neste tecido. Redução significativa do mofo-cinzento, em comparação com parcelas não tratadas, foi observada quando o antagonista era aplicado nos estágios de queda de pétalas e frutos verdes. Entretanto, o antagonista mostrou pouco impacto na redução do mofo-cinzento e baixa consistência nos diferentes experimentos.

Considerando os resultados no seu todo, pode-se dizer que a epidemiologia de *B. cinerea* na cultura do morangueiro varia de um sistema de cultivo a outro. Em morangueiro de cultivo perene, tem sido considerado que restos culturais do próprio morangueiro são os que determinam o potencial de inóculo de *B. cinerea* , estando correlacionado com a incidência do mofo-cinzento nos frutos. No sistema de cultivo anual, verificou-se que plantas de morangueiro contribuem muito pouco na formação de restos culturais e também é baixa a carga de inóculo formada neste substrato para infecção de flores e frutos (Capítulo 2). Por outro lado, quando frutos doentes foram deixados na parcela, o aumento da carga de inóculo para infecção de flores ou frutos causou aumento na incidência do mofo-cinzento na colheita

(Capítulos 5 e 6). Neste caso, conclui-se que a remoção de restos culturais no morangueiro de cultivo anual não resultará no controle do mofo-cinzento, ao passo que a remoção de frutos doentes pode reduzir a incidência da doença, especialmente em condições climáticas favoráveis à infecção. Verificou-se também que a maioria dos sintomas de mofo-cinzento iniciam sob sépalas com mais de 50% dos casos estando presentes pétalas. Isto sugere que a infecção direta no fruto é um mecanismo importante no desenvolvimento do mofo-cinzento, especialmente na presença de pétalas aderidas a superfície do mesmo. O evolução de maturação dos frutos de cultivares de morangueiro utilizadas atualmente, de superfície para seu interior, pode estar facilitando este processo de infecção direta de *B. cinerea* sobre os frutos.

O uso do antagonista *U. atrum* mostrou efetivo controle do mofo-cinzento em cinco dos oito experimentos conduzidos (Capítulos 3 e 6). Por outro lado, o tratamento com a remoção dos restos culturais não reduziu a incidência do mofo-cinzento em comparação com a testemunha (Capítulo 3). Isto mostra que a exclusão de *B. cinerea* nos restos culturais pelo uso de antagonistas ou de outro meio não resultará em eficiência no controle da doença (Capítulo 4). Múltiplas aplicações do antagonista durante a floração mostrou melhor controle da doença em intervalos mais curtos, podendo a dosagem, neste caso, ser reduzida a 0.5×10^6 conidia ml^{-1} que é um quarto da inicialmente usada. Considerando o modo de ação de *U. atrum*, que é por competição do substrato, o antagonista deve alcançar o nicho de competição nas partes senescentes das flores em tempo hábil, isto é, antes ou ao mesmo tempo do patógeno, afim de possibilitar efetiva exclusão do mesmo.

Em condições subtropicais do Brasil, o morangueiro pode ser considerado de cultivo anual, o qual permanece de 80 a 140 dias no campo, para produção de frutos. Em Santa Catarina, como na maioria de outros estados, o morangueiro é cultivado em pequena escala, integrando o sistema de policultivo na maioria das vezes de propriedade familiar. Este desenho de propriedade é altamente favorável em facilitar a conversão para uma agricultura ecológica, orgânica e natural. Neste processo de mudança, o manejo de doenças requer o desenvolvimento de tecnologias harmonizadas com princípios ecológicos e aspectos socio-econômico

na adoção de novos conhecimentos. O estímulo do controle biológico natural deve ser preferido. Entretanto, a reprodução massiva e a subseqüente aplicação de um antagonista nativo pode oferecer uma ferramenta de intervenção temporária e deve ser preferida no lugar da introdução de um novo agente de controle biológico. O aumento da população de antagonistas nativos, tem menor possibilidade de provocar impactos negativos sobre a microflora nativa do que um antagonista importado

Concluindo, restos culturais no cultivo anual do morangueiro tem baixa possibilidade de influir no desenvolvimento do mofo-cinzento dos frutos. Antagonistas nativos podem oferecer uma alternativa de controle biológico durante o processo de conversão em direção a sistemas de produção ecológica; porém, o uso destes agentes dever levar em consideração a não necessidade de reaplicações do referido antagonista.

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Pedro Boff was born on 7 of October 1958, in Capela Santa Lúcia, Antônio Prado, State of Rio Grande do Sul (RS), Brazil. He grew up and started work in his parent's farm until the age of 16 years, when he moved to Porto Alegre, RS, to complete secondary school. From 1980 till 1984, he did graduate school in the Faculty of Agronomy at Federal University of Pelotas, RS, Brazil. In 1987, he completed his Degree of Master in Science, in Plant Pathology, at Federal University of Viçosa, Minas Gerais State, Brazil. Since 1987, he has been working as researcher at Agricultural Research and Rural Extension Agency of Santa Catarina State (EPAGRI), Brazil. In March of 1997, he started his Philosophy Doctor thesis research at Wageningen University and Research Centre, the Netherlands.

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