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Comparative analysis of the role of substrate specificity in biological control of *Botrytis elliptica* in lily and *B. cinerea* in cyclamen with *Ulocladium atrum*

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Abstract

Biological control of *Botrytis* spp. by the fungal antagonist *Ulocladium atrum* is based on their interaction in plant tissue. *U. atrum* is effective against *B. cinerea* in commercial cyclamen crops but not effective against *B. elliptica* in lily crops. Based on the necrotrophic nature of the *Botrytis* spp. and the saprophytic nature of *U. atrum* it is hypothesised, and experimentally confirmed, that the interaction between *Botrytis* spp. and *U. atrum*, resulting in a biocontrol effect, only takes place in necrotic plant tissue. The role of necrotic tissue in the epidemiology of *B. cinerea* in cyclamen and *B. elliptica* in lily was found to be different. Removal of symptomless senescing leaves resulted in a significant reduction of the area under the disease severity progress curve (AUDPC) for *B. cinerea* in cyclamen but had no effect on the disease severity in lily. *U. atrum* applications significantly reduced *B. cinerea* AUDPC values in cyclamen but were less efficient than the removal of senescing leaves. In lily, disease severity was not affected by applications of *U. atrum*. It is concluded that necrotic cyclamen tissue, not killed by *B. cinerea*, plays an important role in the onset of disease. Colonisation of this tissue by *U. atrum* prevents saprophytic colonisation of those leaves by *B. cinerea*. In contrast, conidia of *B. elliptica* directly infect healthy lily leaf tissue. *U. atrum* applications aimed at blocking the infection pathway from a saprophytic base are therefore not effective against *B. elliptica*. Control options based on competitive interactions in and around *B. elliptica* lesions resulted in a reduced production of conidia by *B. elliptica* but proved ineffective against disease development. The potential of *U. atrum* as a biocontrol agent against *Botrytis* spp. and possibly against other necrotrophs appears to be determined by the competitive saprophytic ability of the antagonist in mutual substrates of pathogen and antagonist and by the role of these substrates in disease epidemiology.

Introduction

Botrytis spp. cause economically important diseases in numerous greenhouse and field crops. *Botrytis elliptica* (Berk.) Cooke, the causal agent of 'lily fire' has been responsible for economically significant losses in lily (*Lilium* spp.) production (Doss et al., 1984). *Botrytis cinerea* Pers.:Fr is a major pathogen in many vegetable, flower and fruit crops. It causes leaf rot in cyclamen (*Cyclamen persicum* L.) and is considered to be one of the major pathogens in this crop.

At present, the control of *Botrytis* diseases is based on the frequent use of fungicides. However, *Botrytis* spp. have shown a great potential to develop fungicide resistance (Gullino, 1992; Migheli et al., 1990). In addition, an increasing concern about the effects

of pesticide residues on the environment and human health (Jansma et al., 1993) leads to an increasing number of restrictions on the use of pesticides. Biological control offers an environmentally friendly supplement or alternative to chemical control.

The saprophytic fungal antagonist *Ulocladium atrum* Preuss, isolate 385, is capable of suppressing sporulation of *Botrytis* spp. in necrotic tissue of several plant species (Köhl et al., 1995b,c). In onion, reduction of sporulation of *Botrytis* caused a delay in the disease build up (Köhl et al., 1995a). However, very different results have been achieved in biocontrol experiments where conidial suspensions of *U. atrum* were applied against *B. elliptica* or against *B. cinerea*. *U. atrum* suppresses both *Botrytis* spp. in sterilized necrotic leaf tissue under laboratory and field conditions (Köhl et al., 1995b,c). In commercial cyclamen crops, *U. atrum* can be as effective against *B. cinerea* as standard fungicide applications (Köhl et al., 1998; 2000). However, *U. atrum* applications against *B. elliptica* in field-grown lily crops have not markedly affected lily fire epidemics (Köhl, unpubl.).

Biological control of *Botrytis* spp. by *U. atrum* depends on the interaction between the fungi in plant tissue. This interaction can only take place within a mutual substrate. The outcome of this interaction depends on initial conditions, competitive abilities of pathogen and antagonist within the specific substrate and the environment. *U. atrum* is a saprophyte, typically found on decaying organic material and is not considered to be pathogenic to lily or cyclamen. Necrotrophic pathogens such as *Botrytis* spp. colonise plant tissue killed either by the pathogen itself or by other factors. Sporulation occurs exclusively on necrotic tissue. The host range of *B. elliptica* is limited to lily (*Lilium* spp.) and a few alternative hosts (MacLean, 1948). Conidia and mycelium of *B. elliptica* infect healthy lily tissue (Beyma thoe Kingma and van Hell, 1931; Doss et al., 1984). In contrast, the host range of *B. cinerea* includes over 200 species (Jarvis, 1977; 1980a) and the infective capabilities of conidia and mycelium of *B. cinerea* vary with the host, physiological status of the host tissue and presence or absence of exogenous nutrients at the infection site (van den Heuvel, 1981; Schlösser, 1978). Therefore, the presence and identity of a mutual substrate for *U. atrum* and *Botrytis* spp. will depend on the crop, crop age and the species of *Botrytis* involved and hence will be a key factor in biological control.

In this paper, two pathosystems, *B. cinerea* in cyclamen and *B. elliptica* in lily, are compared in order to explain the different biocontrol capabilities of the antagonist. It is hypothesised that necrotic tissue constitutes the mutual substrate for *B. elliptica* and *U. atrum* in a lily crop and for *B. cinerea* and *U. atrum* in a cyclamen crop. Experiments focus around the role of necrotic tissue in the onset of disease for *B. cinerea* in cyclamen and *B. elliptica* in lily. The suitability of necrotic tissue as a mutual substrate was confirmed in laboratory experiments. Availability of necrotic tissue in lily and cyclamen crops was then studied in field-grown lily and greenhouse cyclamen crops. Symptomless senescing tissue was manually removed to exclude the pathogens from necrotic tissue. The effect of this treatment on epidemics caused by both *Botrytis* spp. was compared to the actual effect of antagonist applications in field-grown lilies and greenhouse cyclamen crops.

Materials and methods

Fungal cultures and conidial suspensions

B. cinerea isolate 700, isolated from gerbera, and *U. atrum* isolate 385, isolated from necrotic onion leaf were grown on oatmeal agar (20 g of milled oats, 15 g of agar and 1 l of tap water) for 2 and 4 weeks, respectively, at 20 °C in the dark. *B. elliptica* isolate BE9401, isolated from lily, was cultured on malt extract agar (Oxoid CM59) for 2 weeks at 18 °C under continuous light from an 18 W black-light and an 18 W cool-white fluorescent tube. A conidial suspension of each fungus was prepared by flooding the cultures with sterile tap water containing 0.01% Tween 80, gently rubbing the colonies with a rubber spatula, and filtering the suspension through 200 μm nylon gauze. Large quantities of *U. atrum* 385 inoculum for use in greenhouse and field experiments were produced on oat grains in autoclavable spawn bags (type 3LS, van Leer Ltd., Poole, Dorset, UK) incubated for 4 weeks at 20 °C in the dark (Köhl et al., 1998). Conidial suspensions were prepared by agitating the grains in chilled tap water containing 0.01% Tween 80 in a small washing machine (Nova MW 100; Nova, Maastricht, The Netherlands) and filtering the suspension as described. Conidial concentrations of all suspensions were estimated with the aid of a haemocytometer and diluted as required.

Substrate specificity experiments

Substrate specificity of *B. elliptica*, *B. cinerea* and *U. atrum* was studied on five tissue types of lily (*B. elliptica*) and cyclamen (*B. cinerea*): (1) healthy leaf tissue (green); (2) senescing leaf tissue (yellow but turgid); (3) necrotic leaf tissue (yellow-brown lacking turgor); (4) necrotic leaf tissue pre-colonised by *U. atrum;* and (5) necrotic leaf tissue pre-colonised by *B. cinerea* (cyclamen leaves) or *B. elliptica* (lily leaves). Plants of asiatic hybrid lily cv. Mont Blanc, which is highly susceptible to *B. elliptica*, were grown in a greenhouse at 18 °C. Cyclamen plants (*Cyclamen persicum* L.) cv. Super Serie, which is susceptible to *B. cinerea*, were grown in a greenhouse at 20 °C. In both crops, no additional lighting was provided and flower buds were removed to prevent pollen deposition on the leaves, which is known to enhance infection by *B. cinerea* (Chou and Preece, 1968). No symptoms of *Botrytis* spp. infections or other diseases occurred.

To produce pre-colonised tissue, symptomless leaves were picked from lily and cyclamen plants and dried slowly at room temperature (Köhl et al., 1995c). The leaves were sealed in small plastic bags and sterilised by gamma irradiation (4 Mrad). To colonise these leaves with *U. atrum*, *B. cinerea* or *B. elliptica*, tissues were rehydrated, sprayed with conidial suspensions (1×10^6 conidia/ml) of the respective fungi, placed on a sterile grid over two sterile filter papers (8 cm in diameter) moistened with 1.5 ml of sterile water in a sterile Petri dish (9 cm in diameter) and incubated at 18 °C in the dark for 48 h.

Following the preparation of the pre-colonised leaves, healthy, senescing and necrotic leaves from lily and cyclamen plants were placed in petri dishes. These tissues and the pre-colonised tissues were sprayed to incipient run-off with conidial suspensions (1×10^6 conidia/ml) of *B. elliptica* (lily tissues only) or *B. cinerea* (cyclamen tissues only) or *U. atrum* or sterile tap water containing 0.01% Tween 80 using an atomizer (Desaga, Heidelberg, Germany) and incubated at 18 °C in the dark for 48 h. Inoculation resulted in average conidial densities of 85 conidia/mm^2 as determined by direct microscopical observations. Three leaves in one Petri dish were used for each host-tissue type-inoculant combination.

After incubation, leaves were examined on a dissecting microscope for lesions, external mycelium and sporulation. Internal mycelial colonisation of the leaves was visualised according to Kessel et al. (1999). One tissue sample, about 3 × 5 mm, was cut from each leaf, fixed in 3% paraformaldehyde in PBS (phosphate buffered saline solution), transferred to a graded series of sucrose solutions with increasing concentrations of 5%, 10%, 20%, 30%, 40% and 50% in PBS and sectioned using a cryostat. Mycelium of *Botrytis* spp. in the sections was labelled specifically using the monoclonal antibody Bc-KH4 (Bossi and Dewey, 1992) conjugated to the green fluorochrome FITC. Mycelium of *U. atrum* in the sections was labelled specifically using the monoclonal antibody Ua-PC3 (N Karpovich-Tate and FM Dewey, Oxford University, unpubl.) conjugated to the red fluorochrome TRITC. Presence or absence of internal mycelium of *Botrytis* spp. or *U. atrum* was determined in six to eight sections per leaf using fluorescence microscopy. Internal mycelium was recorded as absent (–), a trace of mycelium as +/−, low levels of colonisation throughout the sections as + and abundant mycelium as ++.

Cyclamen greenhouse experiments

Three greenhouse experiments used plants of cyclamen cv. Super Serie. Experiment 1 and part of experiment 2 were used to monitor the occurrence of the tissue types in a commercial cyclamen crop. In experiments 2 and 3, effects of applications of *U. atrum* on grey mould epidemics were examined.

In experiment 1, plants were placed on a table with capillary matting in a commercial greenhouse with a spacing just wide enough to prevent the leaves touching, 140 days after sowing. The experiment was arranged in four untreated, replicate plots, each with 24 plants in four rows of six plants. All plots were located on the same table. Plots were separated by six rows of plants. The number of leaf blades and petioles per plant in each of the substrate classes described above were counted at two-week intervals.

For experiments 2 and 3, plants of cyclamen, not previously treated with pesticides, were obtained from a commercial grower 154 (experiment 2) and 70 days (experiment 3) after sowing. Grey mould severity assessed immediately after arrival revealed an average of 0.1 and 0 leaves per plant with sporulating *B. cinerea* in experiments 2 and 3, respectively. Plants were placed on tables with capillary matting at a spacing just wide enough to avoid contact between the leaves. Greenhouse temperature varied within the 15–25 °C range set as minimum and maximum temperature. Relative

humidity mostly varied between 80% and 90%. No pesticides were used.

Experimental treatments were: (1) spraying the plants with tap water containing 0.01% (vol/vol) Tween 80 every 4 weeks; (2) spraying with a conidial suspension of *U. atrum* (1×10^6 conidia/ml) every 4 weeks; (3) an untreated control; and (4) a treatment in which senescing, symptomless, plant parts were removed at 3–4 day intervals. Necrotic plant parts and plant parts with sporulating *B. cinerea* were left on the plant. Experiment 2 included a fifth treatment in which the plants were sprayed with a conidial suspension of *U. atrum* (1×10^6 conidia/ml) once, coinciding with the first application to other spray treatments. Spray treatments were applied 155, 183 and 211 days after sowing in experiment 2 and at 140, 168, 196 and 224 days after sowing in experiment 3. All plants were sprayed individually on and inside the canopy to reach shielded surfaces such as older leaves, petioles and tuber. This technique is generally used to apply fungicides in commercial cyclamen crops.

In experiment 3, *B. cinerea* was introduced by placing six sporulating cultures (3–4 weeks old) in petri plates (9 cm in diameter) in each of the greenhouse compartments ($\pm 80\ m^3$) when the plants were 166 days old. Three cultures were placed underneath each of the two tables at even spaces. The lids of the petri plates were removed for 48 h to allow aerial dispersal of the conidia.

Experiments 2 and 3 were designed as randomized block experiments with 5 and 4 treatments respectively, each treatment replicated 4 times. Two greenhouse compartments were used, each containing two blocks. Each replicate (plot) contained 25 plants arranged in 5 rows of 5 plants. Plots were separated by 50–60 cm.

The presence of leaf blades and petioles in each of the substrate classes in the untreated plots in experiment 2 was monitored, as described for experiment 1. Grey mould severity was assessed at 2 week intervals as the number of leaves per plant with sporulation of *B. cinerea*. The last assessment in experiment 2 took place three weeks after the previous assessment.

Disease severity per plot was calculated as the average number of leaves per plant sporulating with *B. cinerea*. Log-linear regression models, with replicate and treatment as classifying variables, were fitted to the data for disease severity. The models assume that the variance of the data is proportional to Poisson variance. Models were fitted to the data using the method of quasi likelihood (MacCullagh and Nelder, 1989). Overall effects of replicate and treatment were assessed using F-tests for the ratio of the mean deviance for the particular effect and the mean residual deviance. In case F-tests were significant ($P < 0.05$), treatment means on the log-scale were separated using t-tests ($P = 0.05$). The area under the disease severity progress curve (AUDPC) was calculated as the area under the curve of disease severity (Y-axis) against time (X-axis) and analysed using analysis of variance (ANOVA).

Lily field experiment 1

Bulbs of Asiatic hybrid lilies (*Lilium*) cv. Mont Blanc were planted in April 1998. Eight plots (four rows, 1.5×1 m) of 150 plants were established according to standard practice. Treatments were: (1) removal of symptomless leaves, dead from senescence, at 7-day intervals; and (2) an untreated control. Treatments were arranged in a completely randomised design using four replicates. Treatments and observations were started on 27 May 1998 and stopped on 1 July 1998 when 80% of the leaves were infected by *B. elliptica*.

Disease severity was assessed at weekly intervals as the percentage of leaves with symptoms of *B. elliptica* infection per plant for 10 randomly chosen but fixed plants per plot. Leaves dead from natural senescence were counted in all plants per plot. Each time leaves were removed from the plots assigned to treatment 1, 50 of these leaves, randomly chosen, were incubated in moist chambers at 20 °C under the combined continuous light of three fluorescent tubes (1× TLD 18W/08 and 2× TLD 18W/840) for seven days. After incubation sporulating *Botrytis* spp. were identified based on shape and size of the conidia (Ellis, 1971). Disease severity was analysed for each observation date using ANOVA.

Lily field experiment 2

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

Plots were sprayed at weekly intervals with (1) *U. atrum* conidial suspension (2×10^6 conidia/ml) or

(2) with water containing 0.01% Tween 80. Two starting dates for the spray treatments were included to study the effect of introducing the antagonist in different phases of an epidemic caused by *B. elliptica*. Treatments were started on 5 and 19 August. The last treatment was applied on 9 September. A split–split plot design was used with two spray treatments, two starting dates for the treatments and two sampling positions within each plot. Starting dates were assigned to two main plots. Main plot 1 contained six sub-plots, main plot 2 contained 22 sub-plots. The two spray treatments were assigned randomly to the sub-plots within main plots 1 and 2 with three and 11 replicates, respectively.

On 29 October, after the lily fire epidemic, ten necrotic leaves were collected from the dead plants, from each of two positions (sub-sub-plots) within each sub-plot: the top of the canopy and the lowest leaf layers of the canopy. Sampled necrotic leaves were incubated for five days at 20 °C in moist chambers to allow fungi present to sporulate. Fungal sporulation per leaf was quantified using a dissecting microscope distinguishing between *B. elliptica*, *Ulocladium* spp. and naturally occurring saprophytic *Cladosporium* spp. and *Alternaria* spp. The fraction of leaf covered with conidiophores [0–1] and the relative intensity of the sporulation [0–100%] for each of the genera was assessed visually. Multiplication of the two parameters yields the Sporulating Leaf Area Corrected for Intensity (SPLACI), the relative leaf area sporulating at maximum intensity (Köhl et al., 1998).

To estimate the leaf area available for *U. atrum* colonisation on lily leaves infected by *B. elliptica*, the surface area of fire lesions, yellow streaks, and other tissue types as described above, was monitored using 19 leaves in *U. atrum*-treated plots and 12 leaves in Tween-water treated plots, each with only one newly formed lesion. Length and width of the specific tissue types and of the whole leaf were measured at alternating 3- and 4-day intervals. The surface area of each tissue type per leaf was calculated assuming a rectangular shape for leaf, lesion and yellow streaks.

The average sporulation (SPLACI) per leaf was calculated for each plot and each sampling position for each fungal genus. Angularly transformed sporulation data were then analysed using ANOVA, testing for the main effects of starting date, spray treatment, position in the canopy and their interactions. In case F-tests were significant ($P < 0.05$), LSD tests ($P = 0.05$) were used for testing pairwise differences between the means.

Lily climate chamber experiments

The effect of *U. atrum* on the expansion of lesions caused by *B. elliptica* was studied in mist chambers. Mist chambers were constructed from Lexan (General Electric, Pittsfield, Massachusetts), and measured 1.00 × 0.63 × 1.00 m (L × W × H). Each chamber had an open bottom and a Lexan door and was placed on the perforated shelf of a climate cabinet (Bioclim 1600 SP/+5.45 DU, Weiss Technik, Reiskirchen, Germany). The cabinet allowed control of light periods (intensity and duration), temperature and relative humidity of the air (RH) outside the mist chamber. Mist was produced by an ultrasonic humidifier (Stulz Ultrasonic FN 400 H, Stulz, Hamburg, Germany) and introduced into the mist chamber through a 1-m tube, perforated every 3 cm, mounted to the ceiling of the mist chamber. After mist periods two ventilators mounted in the back wall of the mist chamber were opened to allow the RH within the chamber to decline to the level in the cabinet. Timing and duration of mist and ventilation periods and mist density were controlled by computer (A.J.A. van der Zalm and H.W. Roelofsen, unpubl.).

Bulbs of Asiatic hybrid lilies (*Lilium*) cv. Mont Blanc were grown for 12 weeks in pots in a greenhouse as described. No symptoms of fire or other diseases occurred. Ten leaves per plant were inoculated on the abaxial side with a 3-μL droplet of a *B. elliptica* conidial suspension (5×10^4 conidia/ml) according to Doss et al. (1984). Following inoculation, plants were incubated in a mist chamber in the dark at 100% RH for 24 h. Plants were then allowed to dry, spray-inoculated with conidia of *U. atrum* (1×10^6 conidia/ml) or sterile tap water containing 0.01% Tween 80 to run-off using an atomizer (Desaga, Heidelberg, Germany), dried again and returned to the mist chamber. After inoculation, *U. atrum* conidial densities were 29 and 38 conidia/mm^2 on the adaxial and abaxial side of the leaves, respectively, as determined by direct microscopical observation. Effects of *U. atrum* and temperature on the rate of lesion expansion were determined at 6, 12, 15, 18 and 21 °C.

The experiment was done in two mist chambers, each in a separate climate cabinet. Each temperature setting was sequentially tested in each cabinet following a randomized block design in which the temperature settings were assigned randomly to each cabinet. Each mist chamber contained two blocks, each block contained

six plants to which the two spray treatments were randomly assigned in triplicate.

The length and width of all lesions larger than 1×1 mm was recorded on each plant at 1-day intervals, or at 2-day intervals at 6 °C. Measurements stopped when the lesion reached the edge of the leaf. Expansion rates of each lesion were calculated as the slope of the regression line of length, or width on time. Non-expanding lesions were excluded from the analysis. The average lesion length and width growth rate per plant was subjected to ANOVA, testing for main effects of temperature, treatment and their interaction. The relationship of lesion expansion rate to temperature was tested by fitting a second-order orthogonal polynomal within the ANOVA that included tests of the interaction of the linear and quadratic trend with treatment. In case F-tests were significant ($P < 0.05$), LSD tests ($P = 0.05$) were used for testing pairwise differences between the means. All statistical analyses were done using the statistical package Genstat 5 (Numerical Algorithms Group, Oxford, UK).

Results

Substrate specificity

Lesions occurred on healthy and senescing lily leaves that were inoculated with conidia of *B. elliptica*. Healthy and senescing cyclamen leaves that were inoculated with conidia of *B. cinerea* remained symptomless. External mycelium was found on necrotic lily and cyclamen leaves pre-colonised by *B. elliptica* and *B. cinerea*, respectively, or by *U. atrum*. External mycelium also occurred on necrotic lily and cyclamen leaves inoculated with *B. elliptica* and *B. cinerea*, respectively. Sporulation was only found on necrotic cyclamen leaves pre-colonised by *B. cinerea*.

The abilities of both *Botrytis* species to colonise the various types of plant tissue are clearly different (Table 1). *B. elliptica* conidia established mycelial colonisation in healthy, senescing and necrotic lily leaf tissue. *B. cinerea* did not appear to colonise healthy or senescing cyclamen leaf tissue. However, necrotic cyclamen leaf tissue was colonised. *U. atrum* was only able to colonise dead lily or cyclamen tissue. In tissues already colonised by *U. atrum, B. elliptica* established low levels of mycelium whereas *B. cinerea* was excluded. *U. atrum* established low levels of mycelial colonisation in necrotic lily and cyclamen tissues already colonised by *B. elliptica* or *B. cinerea*, respectively.

Cyclamen greenhouse experiments

Numbers of green leaves per plant increased linearly from 22 to 65 and from 31 to 52 during experiments 1 and 2, respectively. Senescing and necrotic petioles and leaf laminae first appeared about 140 days after sowing (Table 2). Sporulation of *B. cinerea* was typically first found on leaves dead due to senescence.

Table 1. Substrate specificity of *B. elliptica* (Be), *B. cinerea* (Bc) and *Ulocladium atrum* (Ua) for lily and cyclamen tissues. Three non-colonised tissue types and two tissue types pre-colonised during 48 h were inoculated with conidial suspensions of *B. elliptica* (lily), *B. cinerea* (cyclamen) and *U. atrum* (both lily and cyclamen). Mycelium within the tissue was detected immuno-histologically after 48 h incubation at 18 °C in a moist chamber. Classification is based on observations of 16–24 sections per host, inoculant and tissue type combination.

Inoculant	Lily						Cyclamen					
Fungus detected	Water		*B. elliptica*		*U. atrum*		Water		*B. cinerea*		*U. atrum*	
	Be	Ua	Be	Ua	Be	Ua	Bc	Ua	Bc	Ua	Bc	Ua
Tissue type												
Non-colonised												
Healthy	—	—	+/−	—	—	—	—	—	—	—	—	—
Senescing	—	—	+	—	—	—	—	—	—	—	—	—
Necrotic	—	—	+	—	—	++	—	—	+	—	—	+
Pre-colonised												
Necrotic + Ua	—	++	+/−	++	—	++	—	++	—	++	—	++
Necrotic + Be	++	—	++	—	++	+/−	nd	nd	nd	nd	nd	nd
Necrotic + Bc	nd	nd	nd	nd	nd	nd	++	—	++	—	++	+/−

—, No mycelium detected; +/−, Trace of mycelium detected; +, Mycelium clearly present; ++, Mycelium abundantly present; nd, Not Done.

Table 2. Total number of leaves and senescing and necrotic leaf parts in untreated plots of cyclamen greenhouse experiments 1 and 2. In brackets the number of plant parts with sporulating *B. cinerea*. Means in the table are based on counts in 4 replicate plots containing 24 and 25 plants each in experiment 1 and 2, respectively

Experiment	Plant age (days)	Leaves per plant	Senescing leaf parts (plant^{-1})		Necrotic leaf parts (plant^{-1})	
			Laminae	Petioles	Laminae	Petioles
1	140	22.4	0.4 (0.0)	0.4 (0.0)	0.2 (0.0)	0.0 (0.0)
	153	37.4	0.6 (0.0)	0.5 (0.0)	0.4 (0.0)	0.2 (0.0)
	168	46.2	0.9 (0.0)	0.3 (0.1)	0.7 (0.2)	0.5 (0.1)
	182	51.2	2.1 (0.0)	1.0 (0.0)	1.6 (0.6)	1.2 (0.3)
	194	65.2	1.1 (0.0)	0.8 (0.1)	3.8 (1.3)	3.1 (0.5)
2	157	30.7	0.6 (0.0)	0.1 (0.0)	0.5 (0.1)	0.4 (0.2)
	171	39.3	1.0 (0.0)	0.5 (0.1)	2.2 (1.1)	1.1 (0.6)
	183	44.1	1.1 (0.0)	0.6 (0.0)	2.5 (1.1)	1.4 (0.5)
	197	45.4	1.0 (0.0)	0.6 (0.1)	3.0 (1.2)	1.9 (0.7)
	213	51.6	0.8 (0.0)	0.2 (0.1)	3.4 (1.8)	2.3 (1.7)

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

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

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

Regular removal of senescing, symptomless leaves markedly reduced disease severity (Table 3, Figure 1). *U. atrum* applications reduced AUDPC values but less effectively than removal of senescing leaves. When *B. cinerea* was present in the crop from the start of experiment 2, reduction of the AUDPC values was marginal (Table 3, Figure 1).

Lily field experiment 1

The number of leaves, dead from senescence but otherwise symptomless, was six per plot on 27 May 1998 after which it increased to a stable level of 80–100 leaves per plot in the first week of June and thereafter. These leaves were mostly in the lower layers of the canopy. After incubation in high humidity, *B. cinerea* sporulated on 50% of these leaves. Sporulation of *B. elliptica* was never found. Removal of symptomless lily leaves dead from senescence did not significantly affect the lily fire epidemic at any time.

Lily field experiment 2

Numerous primary infections of *B. elliptica* were first found on 16 August. Small amounts of necrotic tissue (Figure 2), associated with the yellow streaks, were observed distally from and outside the *B. elliptica* lesion. No indications were found that *U. atrum* treatments affected yellow streak formation on infected leaves. Fire lesions often almost covered the complete leaf with *B. elliptica* sparsely sporulating on the lesion.

On dead leaves collected and incubated after the lily fire epidemic, dense sporulation of *Ulocladium* spp. and other saprophytes occurred on small areas, at the tip and or base of the leaf but outside the fire lesions. Regular *U. atrum* applications significantly increased sporulation of *Ulocladium* spp. and significantly reduced sporulation of *B. elliptica* and *Cladosporium* spp. on these leaves (Table 4). Other saprophytes (mainly *Alternaria* spp.) were too rare to be included in the analysis. Different starting dates of spray treatments did not affect the sporulation of *B. elliptica* and *U. atrum*. *Cladosporium* spp. were significantly less abundant in plots within the main plot assigned to starting date 2. *B. elliptica* and *U. atrum* were significantly more

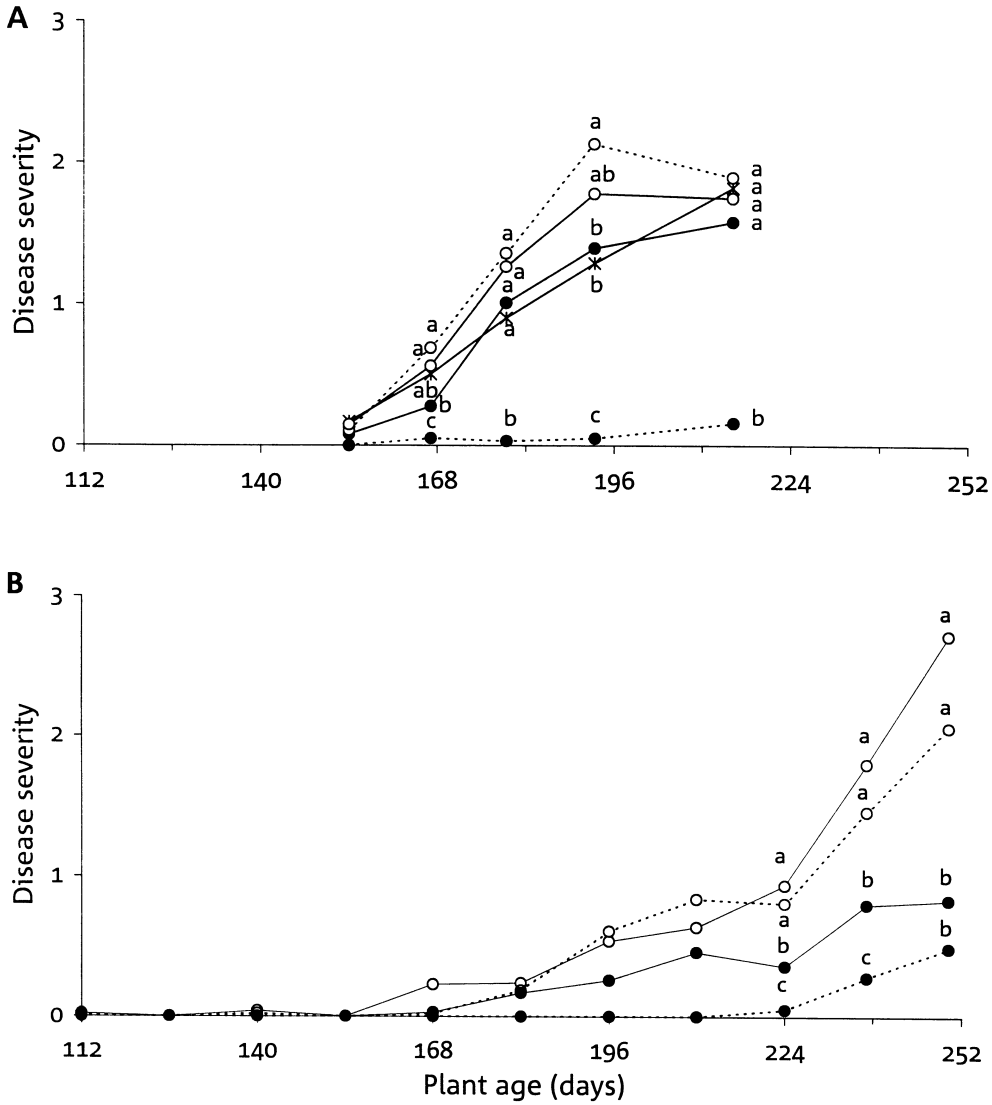

Figure 1. Effect of *Ulocladium atrum* applications and the removal of senescing, symptomless leaves on the disease severity caused by *Botrytis cinerea* in cyclamen cv. Super Serie in greenhouse experiments 2 (A) and 3 (B). Disease severity is defined as the average number of leaves with symptoms of *B. cinerea* per plant. (–○–): untreated control; (- -○- -): water containing 0.01% Tween 80 applied at 4 week intervals; (–●–): *U. atrum* (1×10^6 conidia/ml) applied at 4-week intervals; (–*–): *U. atrum* (1×10^6 conidia/ml) applied once, coinciding with the first treatment of the other spray treatments; (- -●- -): senescing, symptomless leaves removed at 3–4 day intervals. Data of experiment 3 were analysed starting at a plant age of 224 days. Values from the same assessment date with a common letter do not differ significantly ($P < 0.05$) according to log-linear regression followed by t-tests.

abundant in the highest canopy layers than in the lower canopy layers. At both levels, *U. atrum* applications had a similar effect on *B. elliptica* as indicated by the absence of a Treatment/Position interaction for these two species in the analysis of variance. *Cladosporium* spp. were equally abundant in the highest and lowest canopy layers in the control treatment. *U. atrum* applications were significantly more suppressive to the sporulation of *Cladosporium* spp. in the higher canopy layers than in the lower canopy layers.

Detailed observations revealed a distinct spatial separation between sporulation of *B. elliptica* within the

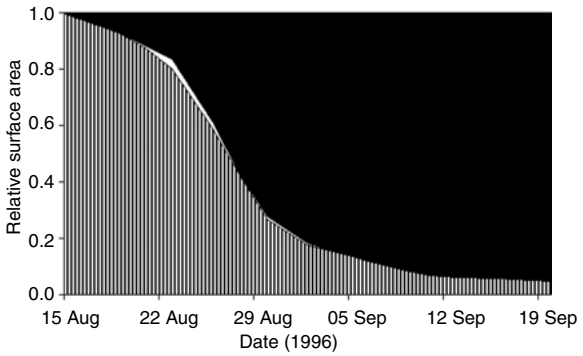

Figure 2. Relative surface area of different tissue types (substrates) present in lily leaves infected by *Botrytis elliptica* during a lily fire epidemic in field experiment 2. Thirty one leaves with a total area of 222 cm^2 were monitored during the growing season. Black area = *B. elliptica* lesion; Hatched area = healthy or senescing tissue; White area = necrotic tissue outside the lesion.

Table 4. Effect of *U. atrum* applications on the sporulation of *B. elliptica*, *Ulocladium* spp. and *Cladosporium* spp. on lily leaves infected by *B. elliptica* in lily field experiment 2

Effect	SPLACI (%)[1]		
	B. elliptica	*Ulocladium* spp.	*Cladosporium* spp.
Starting date			
Starting date 1	10.9	4.4	8.5 a
Starting date 2	10.3	4	5.1 b
Treatment			
U. atrum	8.3 a	13.8 a	2.7[2]
Control	12.7 b	0.1 b	9.9[2]
Position[3]			
High	14.8 a	5.8 a	3.6[2]
Low	6.7 b	2.7 b	8.3[2]
Treatment position			
U. atrum			
High	12.6	17.7	0.7 a
Low	4.8	10.3	5.9 b
Control			
High	17.1	0.3	8.6 bc
Low	8.9	0	11 c

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

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

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

[3]High = top leaf layers of the canopy; Low = lowest leaf layers of the canopy.

former lesion and sporulation of *U. atrum* and other saprophytic fungi outside the former lesion. At the interface of sporulation between *B. elliptica* and saprophytic fungi, the species came into close contact and no inhibition zone was seen.

Climate chamber experiment

At all temperatures tested, *U. atrum* treatment did not significantly affect the longitudinal or transversal lesion growth rate of fire lesions as compared to the control treatment. *U. atrum* did also not influence lesion establishment as for both treatments the number of established lesions did not differ significantly. Longitudinal growth of fire lesions was approximately twice as fast as transverse growth. Longitudinal lesion growth rates were: 4.3, 5.2, 7.9, 7.2 and 9.1 mm/day for 6, 12, 15, 18 and 21 °C, respectively. Transverse lesion growth rates were: 1.4, 2.6, 3.4, 3.9, and 5.2 mm/day for 6, 12, 15, 18 and 21 °C, respectively. Longitudinal and transverse growth rates increased linearly with temperature, in the temperature range employed, with 0.32 and 0.24 mm/day per unit of temperature (°C), respectively.

Discussion

Successful infections of healthy, green, plant tissue by *Botrytis* spp. result in spreading lesions, non-spreading lesions or in quiescent infections as determined by host resistance, nutrient and water status of the infected tissue and climatic and cultural factors (Jarvis, 1980b). Conidial inoculations of healthy lily leaves with *B. elliptica* resulted in spreading lesions. Conidial inoculations of healthy cyclamen leaves with *B. cinerea* did not result in lesion formation. Histological evidence for quiescent infections was not found (Table 1). Conidial inoculations of healthy cyclamen or lily tissue with *U. atrum* did not cause any symptoms nor quiescent infections (Table 1). On senescing tissue, results of the infection experiments were similar to those on healthy tissue. Schlösser (1978) also found it difficult to establish *B. cinerea* infection on cyclamen leaves after inoculation with agar discs overgrown with mycelium. Resistance was attributed to an unidentified inhibitory compound present, at decreasing concentrations, in young leaves, old leaves, senescing leaves and petioles, respectively.

Mycelia of *B. elliptica*, *B. cinerea* and *U. atrum* interact in necrotic tissue but not in healthy or senescing

tissue. In necrotic tissue not killed by *Botrytis* spp., the level of control of *B. elliptica* or *B. cinerea* using *U. atrum* is a function of the available inocula and the environment (Andrews, 1992). In lesions caused by *Botrytis* spp., however, the available necrotic tissue is already colonized by the pathogen and the data on pre-colonised tissue in Table 1 suggest that *U. atrum* does not co-colonise or re-colonise necrotic tissue already colonised by *B. elliptica* or *B. cinerea*. Results from the climate chamber experiment with *B. elliptica* on lily indicate that if co-colonisation of *B. elliptica* lesions by *U. atrum* does occur, it does not significantly affect lesion expansion. Under field conditions, however, *U. atrum* did colonise necrotic tissue available on lily leaves infected by *B. elliptica* and reduced *B. elliptica* sporulation (Table 4). The distinct spatial separation between sporulation of *B. elliptica* within the primary lesion and sporulation of *U. atrum* and other saprophytic fungi outside it suggest that *U. atrum* colonises necrotic leaf tissue available outside (Figure 2) rather than inside the lesion.

In fields, necrotic lily tissue not killed by *B. elliptica* is present in the lowest leaf layers during most of the growing season (Figure 2, lily field expt. 1). Sporulation of *B. elliptica* on this tissue was never found, not even after incubation under humid conditions. The removal of symptomless necrotic lily leaves, dead from senescence never affected lily fire severity. We conclude that this type of necrotic tissue does not play a significant role in lily fire epidemiology. Conidia of *B. elliptica* directly infect healthy lily tissue (Beyma thoe Kingma and van Hell, 1931; Table 3) and the pathogen thus bypasses an infection pathway that might be effectively blocked by *U. atrum*.

On infected leaves, applications of *U. atrum* reduced sporulation of *B. elliptica* and *Cladosporium* spp. by approximately 30% and 70%, respectively (Table 4) but disease severity was unaffected. Apparently, the level of sporulation suppression was not enough to affect the lily fire epidemic significantly. At least three factors may contribute to this effect. (1) Sporulation as measured after the epidemic might not represent sporulation during the lily fire epidemic. (2) *U. atrum* colonises small parts of the yellow streaks outside the established, brown necrotic, *B. elliptica* lesions (Doss et al., 1988), only limiting the leaf area available for lesion expansion after most of the damage has been done. (3) Multiple infections of leaves, rapidly expanding lesions and leaf-to-leaf contact infections contribute to dispersal of *B. elliptica* within and between plants and may compensate for a significant loss of conidia.

Necrotic tissue, dead due to senescence, is present in cyclamen crops during the second half of the growing period (Table 2). *B. cinerea* sporulation is typically first observed on this type of leaf. Regular removal of the precursor of this type of tissue, senescing, symptomless cyclamen leaves, resulted in a significant reduction of the AUDPC (Table 3, Figure 2). Apparently, cyclamen leaves, dead from senescence, play an important role in the onset of disease. Saprophytic colonisation of these leaves by *U. atrum* could exclude later saprophytic colonisation by *B. cinerea* and result in a biocontrol effect (Table 3, Figure 1). Mycelial contact infections from *B. cinerea*-colonised necrotic tissue to the petioles, which are the least resistant (Schlösser, 1978), and from petiole to petiole could then generate the typical symptom of infected clusters of leaves.

U. atrum applications against *B. cinerea* in cyclamen were effective in greenhouse experiment 3 but less effective in greenhouse experiment 2. This difference, which contrasts with earlier findings (Köhl et al., 1998; 2000), can be explained by *B. cinerea* being in the crop of experiment 2 before the first application of *U. atrum*.

The necessity for prior colonisation of necrotic tissue by *B. cinerea* in cyclamen is exploited to minimise infection by the pathogen using *U. atrum* applications. During the interaction in necrotic tissue, *U. atrum* benefits from long interaction times, the absence of an escape for the pathogen (e.g., infection of healthy tissue) and protection from adverse abiotic conditions (Fokkema, 1993; Köhl and Fokkema, 1998). The presence of a saprophytic base for infection of healthy tissue was also reported for *B. cinerea* in lettuce (Wood, 1951), tomatoes (Newhook, 1957), grapes (Dubos et al., 1982) and for *Sclerotinia sclerotiorum* in bean (Boland and Hunter, 1988). *U. atrum* could thus be especially effective against pathogens in such pathosystems. Recently, *U. atrum* has been shown to control *B. cinerea* in grapes effectively under field conditions (Schoene and Köhl, 1999).

This study has identified several factors which determine the biological control potential of *U. atrum* against *Botrytis* spp: (1) a mutual substrate should exist, (2) the antagonist should be able to exclude or outcompete the pathogen in the mutual substrate, and (3) the mutual substrate should play an important role in the epidemiology of the pathogen. This rational approach identifies the target(s) and the aim for antagonist

application, increases knowledge of the pathosystem and allows better timing of actual applications.

As a crop protection agent, *U. atrum* differs from agents directly affecting parasitic fitness (MacKenzie, 1978) such as fungicides and biocontrol agents effective through hyperparasitism or secondary metabolites. *U. atrum* accelerates the degradation of necrotic tissue which is used by the pathogen as a stepping stone towards infection of the cyclamen plant. *U. atrum* therefore controls *B. cinerea* in cyclamen through biosanitation, similar to, but more effective than, the natural saprophytic population.

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