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The survival and saprophytic competitive ability of the *Botrytis* spp. antagonist *Ulocladium atrum* in lily canopies

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Abstract

In 1995, conidia of *Ulocladium atrum* were applied to a canopy of green lily (*Lillium* spp.) leaves in order to investigate its survival, colonisation of artificially induced necrotic leaf tissues and competitive ability against *Botrytis* spp. and naturally occurring saprophytes. *U. atrum* conidia density cm^{-2} at the top and middle canopy levels was not significantly different following application of the antagonist with a propane powered backpack sprayer. In repeat experiments, conidia density on leaves at the lower canopy level was 18% to 20% of that deposited onto leaves at the top of the lily canopy. There was a significant ($P < 0.001$) linear decline of *U. atrum* conidia over time and after 21 days conidia density had declined by up to 73%. Germination of *U. atrum* on green leaves in the field reached a maximum of 81%, seven days after antagonist application. Conidial viability, measured as germination potential, declined slightly (100% to 88%) after seven days exposure to field conditions but there were no further changes in the germination potential even after 21 days of field exposure. The germination potential was not affected by canopy level. The ability of surviving *U. atrum* conidia to colonise necrotic tissues, artificially induced with paraquat, was measured. *U. atrum* colonisation was consistently highest on necrotic leaves at the top level of the canopy and consistently lower on leaves from the bottom canopy level. Necrotic leaf colonisation by *U. atrum* decreased over time from 51% (necrosis induced immediately after antagonist application) to 21% when necrosis was induced 21 days after antagonist application. A significant ($P < 0.001$) linear relationship ($R^2 = 0.713$) between colonisation of necrotic tissues and conidia density prior to induction of necrosis was detected. When necrosis was induced immediately after antagonist application, *U. atrum* outcompeted commonly occurring saprophytic *Alternaria* spp. and *Cladosporium* spp. The ability of *U. atrum* to significantly reduce colonisation by *Alternaria* spp. was maintained for up to 21 days. *Botrytis* spp. did not occur in these field experiments. It was concluded that *U. atrum* had the ability to survive and persist in the phyllosphere for up to 21 days in the field and provided further evidence that *U. atrum* has the necessary survival characteristics to be a successful biological control agent of *Botrytis* spp.

Introduction

Botrytis elliptica (Berk.) Cooke, the causal agent of 'lily fire' has been responsible for economically significant yield losses in bulb production systems (Doss et al., 1984). Disease control is based upon repeated applications of fungicide, but the sustainability of this practice is questionable for two reasons. First, fungicide resistance has emerged in populations of *B. cinerea* (Gullino, 1992) and *B. elliptica* (Chastagner and Riley, 1990; Migheli et al., 1990). Second, there are increasing public concerns about the effects of pesticide residues on the environment and human health (Jansma et al., 1993). Biological control of diseases caused by *Botrytis* spp. may be a more acceptable alternative or supplement to the use of fungicides.

Antagonists may interfere with *Botrytis* spp. at three different stages of the life cycle of the pathogen. (1) Survival structures such as sclerotia may be the primary source of pathogen inoculum and these can be colonised and destroyed by antagonists such as *Trichoderma* spp. (Köhl and Schlösser, 1989). (2) Pathogen infection of healthy tissues may be prevented by bacteria (Leifert et al., 1995; Swadling and Jeffries, 1996), yeasts (Redmond et al., 1987) or filamentous fungi (Elad et al., 1993). (3) Spread of the disease between growing seasons or within a crop during a polycyclic epidemic may also be reduced by antagonists which colonise necrotic plant tissues. These tissues are often the principle substrate on which necrotrophic pathogens such as *Botrytis* spp. sporulate. Successful microbial suppression of the initial inoculum of several necrotrophic pathogens such as *Pyrenophora tritici-repentis* (Pfender et al., 1993) and *Venturia inaequalis* (Heye and Andrews, 1983) has been reported.

Suppression of sporulation of *B. cinerea* and *B. squamosa*, the causal agents of onion leaf spot, significantly reduced the rate of epidemic development in the field (Köhl et al., 1995a). The strategy of biologically suppressing sporulation of *Botrytis* spp. has two principle advantages. (1) The interaction time between the saprophytic antagonist and the pathogen in necrotic tissues is in the order of days compared to the possible interaction time of a few hours which may occur during infection of healthy tissues (Nair and Allen, 1993). (2) The basis of antagonism by saprophytes especially against the saprophytic stages of necrotrophic pathogens in necrotic tissues appears to be based on nutrient competition (Fokkema, 1993). The risk of resistance development in *Botrytis* spp. populations against a nutrient competitor is unlikely. Potentially, there is a much higher risk of resistance development when the mode of biocontrol is based upon the production of antibiotics by micro-organisms as was shown in experiments with *Bacillus subtilis* for *B. cinerea* control (Li and Leifert, 1994).

Antagonists applied to necrotic or green leaf tissues in the field must be well adapted to these ecological niches since both are characterised by large and rapid fluctuations in temperature, water availability and radiation. Therefore, effective antagonists must not only survive periods unfavourable for growth and development, but must also have the capacity to grow rapidly during the limited periods favourable for growth in order to colonise and utilise the substrate. The fungal antagonist *Ulocladium atrum* G. Preuss suppressed sporulation of *Botrytis* spp. on necrotic leaf tissues under continuously moist conditions and when leaf wetness periods were repeatedly interrupted in controlled environments to simulate field conditions (Köhl et al., 1995c). Recent research suggests that antagonism by *U. atrum* was most likely based on competition and not the production of antibiotics (Köhl et al., 1997).

In separate studies, *B. cinerea* colonisation of necrotic lily leaves exposed to field conditions was consistently reduced by 90% on *U. atrum* treated tissues in comparison to non-treated leaves in nine independent experiments (Köhl et al., 1995b). In addition, this antagonist was consistently able to survive and colonise necrotic lily leaves in the presence of other competing saprophytes when exposed to field conditions. Survival and colonisation of necrotic tissues was not restricted to lily and onion tissues. When *U. atrum* was applied to necrotic kiwifruit leaf disks and exposed to field conditions in New Zealand orchards, the tissues became colonised by antagonists and there was a significant reduction of *B. cinerea* sporulation (Elmer et al., 1995).

When *U. atrum* conidia are sprayed onto a crop, a large proportion of the antagonist inoculum is deposited onto healthy green tissues. Since the primary target is necrotic leaf parts, inoculum of the antagonist may be considered 'lost' from the system. However, green leaves or parts thereof will potentially become necrotic as a result of natural senescence, pathogen infection, or other biotic or abiotic factors such as freezing injury. If viable antagonist inoculum is present on green tissues in sufficient quantities just prior to the onset of necrosis then rapid colonisation by the antagonist of the necrotic tissue may exclude the pathogen and or other competitive saprophytes from that substrate. Therefore in a biological control system aimed at colonisation of necrotic tissues the level of viable antagonist inoculum on green leaves may be of significant importance.

The objective of our study was (1) to investigate the fate of *U. atrum* inoculum sprayed onto green lily leaves under field conditions and (2) to determine its ability to colonise freshly induced necrotic leaf tissues after exposure on green leaf surfaces. Spore density on the green leaf surface, survival and germination of *U. atrum* conidia and the superficial growth of germ tubes was followed for a period of three weeks. Leaf necrosis was artificially induced and colonisation of that substrate by *U. atrum*, *Botrytis* spp. and naturally occurring saprophytes was quantified.

Materials and methods

Antagonist inoculum

Ulocladium atrum isolate 385 G. Preuss, isolated originally from a necrotic onion leaf tip (Köhl et al., 1995a), was used in these studies. Storage cultures of *U. atrum* were preserved in 5 ml sterile vials containing a cryopreservative fluid (Protect, Technical Service Consultants Ltd, UK) at −80 °C and stored until required. Conidial inoculum of *U. atrum* was prepared from four week old cultures which had been grown on moistened whole oats (autoclaved twice for 45 min at 120 °C, 100 kPa) in the dark at 18 °C in sterile autoclave culture bags (Type 3L3, Silvan Distributors, Horst, The Netherlands). A suspension was prepared by agitating *U. atrum* colonised oats in a fine mesh nylon bag thoroughly in approximately 500 ml chilled (4 °C) tap water (plus 0.01% Tween 80). The resultant suspension was filtered through sterile nylon gauze (mesh of 200 μm). The concentration was determined with the aid of a haemocytometer and adjusted to 2×10^6 conidia ml^{-1} with chilled tap water containing 0.01% Tween 80. The suspension was kept at 4 °C for a maximum of 4 h before use.

Field plots and treatments

Bulbs of the Asiatic hybrid lily (*Lilium* cv. Mont Blanc) were planted on 18 April 1995 in two 5 m × 1 m plots with each plot separated by 8 m buffers of sugar beet. The herbicide chloridazon (Pyramin, 650 g a.i. kg^{-1}, BASF, Germany) was applied at 19 April and thereafter no further pesticide applications were made. Prior to anthesis, all flower stems in each plot were removed on 5 July 1995 by hand as normal commercial practise in bulb production. The *U. atrum* suspension was applied on 24 July 1995 (Experiment 1) to five randomly chosen replicate microplots in a 5 m × 1 m plot using a propane powered backpack sprayer (AZO, Ede, The Netherlands) with a single nozzle with a pressure of 250 kPa. Tap water (plus 0.01% Tween 80) was applied to five randomly chosen replicate control microplots also located in the same plot. Each microplot consisted of at least nine individual plants of equal height and growth stage. The experiment was set up as a 2 * 3 * 4 factorial split plot design with treatment factors that were; *U. atrum* application (plus and minus), canopy level (top, middle, bottom) and time of inducing necrosis after *U. atrum* application (0, 7, 14 and 21 days) with five replications (microplots). The experiment was repeated in a separate 5 m × 1 m plot on 31 July 1995 (Experiment 2). All treatments were sprayed in the evening and a metal frame (50 cm × 30 cm × 30 cm) surrounded by polythene plastic was carefully placed around each microplot at the time of application to minimise drift from one microplot to another.

Microclimate measurements

Temperature and relative humidity (RH) of the air were measured with an electronic sensor (airprobe YA-1OO-hygromer, Rontronic AG, Bassersdorf, Switzerland) positioned at a height of 35 cm in an onion field approximately 50 m away from the treated plots. Rainfall was recorded with a rain gauge placed in the same field. Three leaf wetness sensors (Köhl et al., 1995b) were used to monitor leaf wetness of necrotic lily leaves in the lily canopy in an additional microplot. All data were recorded at 30-min intervals and stored in a data-logger (Delta-T Logger, Delta-T Devices Ltd. Burwell, Cambridge, UK).

Survival of U. atrum *conidia on green lily leaves*

Sampling and sample processing

One hour, 7, 14 and 21 days after *U. atrum* application, one green leaf was randomly selected per replicate from each of the top, middle and bottom level of a single plant, located approximately 25 cm, 15 cm and 5 cm respectively from the soil. Each leaf was placed into a sterilised plastic vial and samples were immediately returned to the laboratory. Sampled leaves were then cut in two along the axis of the main vein with the aid of a scalpel. In order to determine the density (number of conidia cm^{-2} leaf surface) and percentage of *U. atrum* conidia which had germinated on the leaf surface in the field (field germination), one half of the leaf was placed into an ammonia fumigation chamber to terminate all microbial growth. The fumigation chambers consisted of sterile plastic petri dishes (90 mm diameter) to which was added five drops of household ammonia (4.8% NH_4OH in water) to a single layer of filter paper (Whatman, 50 mm diameter) attached to the lid of the chamber. To determine the percentage of viable *U. atrum* conidia on the leaf surface (germination potential), the other half of the leaf was placed into a high humidity chamber. The chambers consisted of sterile plastic petri dishes (90 mm diameter) containing two sterile filter papers (Whatman, 80 mm diameter), moistened with 1 ml sterile tap water. Growth was terminated after 18 h incubation at 18 °C in the dark by the addition of ammonia

as described above. All leaf samples were stored in a freezer at − 18 °C until measurement.

Conidia density and germination measurements

Half leaf sections were recut to 1 to 2 cm and placed onto glass microscope slides. A 100 μl aliquot of the fluorochrome Calcofluor White (Fluorescent Brightener 28; F-6259, Sigma; 0.02%, w/v in 1M Tris HCl buffer, pH 8.0) was added to the leaf surface and then examined with a Zeiss Axioskop Fluorescence Microscope (Filter 05, Zeiss, Oberkochen, Germany) using a blue/violet (395 to 440 nm) light source. After staining with Calcofluor, little or no background autofluorescence from plant cells was observed and spore morphology was used to distinguish *U. atrum* conidia from other saprophytic species. The number of conidia in an ocular eyepiece grid (representing 0.25 mm^2 of leaf surface) was counted and the average number of conidia per grid calculated from 10 grids. The first observation was always started at an arbitrarily chosen position on the leaf and the other observations were selected at regular intervals along a diagonal transect across each leaf section and the results expressed as the number of conidia cm^{-2} of leaf surface.

Field germination (%) and germination potential (%) were determined by counting all conidia which had germinated in several fields of view, representing 0.64 mm^2 of leaf surface. The first observation was always started at an arbitrarily chosen position on the leaf and the other observations were selected at regular intervals along a transect across the surface of each preparate. Clusters of conidia were avoided and a minimum of 50 conidia in total were counted. Germination was said to have occurred when the germ tube length was greater than half the diameter of the conidium.

Fungal colonisation of artificially induced necrotic lily leaves

Induction of necrosis

In order to determine the ability of surviving *U. atrum* inoculum on green leaf surfaces to successfully colonise necrotic tissues, a method was developed which induced localised necrosis on tagged green leaves in the field. Zero, 7, 14 and 21 days after *U. atrum* application, necrosis was artificially induced locally on tagged leaves, selected at random from approximately 25 cm, 15 cm and 5 cm from the soil surface, representing the top, middle and bottom levels, respectively. There were four lily plants per replicate and five replicates per treatment. Induction of necrosis was achieved using a desiccant herbicide Gramoxone (200 g l^{-1} paraquat dichloride, Zeneca Agrochemicals, UK). Paraquat has been used in several phytopathology studies as a tool to aid the identification of fungi in host issues (Bannon, 1978; Cerkauskas and Sinclair, 1980). However, paraquat is known to affect some fungal species more than others (Wilkinson and Lucas, 1969) and spore germination may be adversely affected at concentrations as low as 32 μg ml^{-1} paraquat (Michailides and Spotts, 1991). Our preliminary studies indicated that 10 mg l^{-1} paraquat (plus 0.1% Tween 80) was sufficient to induce localised necrosis on healthy green lily leaves but did not affect spore germination (%) or germ tube growth of an isolate of *B. cinerea*, *B. elliptica*, *Alternaria* spp. *Epicoccum purpurascens*, *Ulocladium* spp. or *U. atrum* (Elmer and Köhl, unpublished).

To induce necrosis on healthy green lily leaves in the field, a small piece (1.5 cm^2) of household moisture absorbent cloth was fixed firmly to the underside of tagged leaves and kept in place with the aid of a paper clip. Six hundred μl of paraquat suspension (10 mg l^{-1} plus 0.1% Tween 80) was then carefully pipetted between the cloth and the underside of the leaf. Paraquat treatment was always made after sunset to avoid the absorbent cloth drying too rapidly and the cloth and paper clip were removed 48 h after paraquat application. The first symptoms of tissue necrosis were observed at that stage and complete necrosis of the entire leaf occurred five to seven days after paraquat treatment.

Sampling

Fourteen days after each application of paraquat (applied at Day 0, Day 7, Day 14 and Day 21), one paraquat treated necrotic leaf was removed from each of the top, middle and bottom zones of four plants per replicate with flame sterilised forceps. The four leaves per canopy level per replicate were placed into the same sterile high humidity chambers. The chambers consisted of sterile plastic petri dishes (110 mm diameter) with two layers of filter paper (Whatman, 100 mm diameter) moistened with 3 ml sterile tap water. The humidity chambers were sealed with parafilm and incubated at 18 °C in the dark for two days. The parafilm was then removed and the necrotic leaves incubated for a further seven days (Köhl et al., 1995b). All samples were then sealed in plastic bags and stored at −18 °C.

Measurement of fungal colonisation

The area of necrotic tissue covered with sporulating *U. atrum* and other commonly occurring saprophytes was determined with the aid of a stereo microscope. A 25 mm^2 quadrat was prepared from a clear acetate sheet and placed at random onto the necrotic leaf surface. The area of sporulation in each 25 mm^2 quadrat by *U. atrum*, *Alternaria* spp., *Cladosporium* spp., *Epicoccum* spp., *Stemphylium* spp., and *Botrytis* spp. was then estimated for four quadrats per leaf. Results were expressed as percent coverage of the surface of necrotic tissue.

Statistical analysis

For the *U. atrum* treated microplots, an ANOVA was performed on the observed density counts of conidia after logarithmic transformation using a split-plot model with canopy level, time after *U. atrum* application and their interaction as explanatory variables and random effects for variation between plots, canopy levels within plots and time within canopy levels within plots. The data for percentages of germinated conidia and the percentages of leaf area colonised by *U. atrum* were angular transformed prior to ANOVA using the same split-plot model. Orthogonal polynomials were used to partition the sum of squares for time as well as for the interaction between time and canopy level into the amount that may be explained by a linear relationship of the response with time, the extra amount that may be explained if the relationship was quadratic and the amount represented by deviations from a quadratic polynomial. For the various time intervals separate ANOVA's were performed to percentages of leaf area colonised by *Alternaria* spp. and *Cladosporium* spp., respectively, after angular transformation using a split-plot model with treatment, canopy level and their interaction as explanatory variables and random effects for variation between plots and variation between canopy levels within plots. F-tests for the variance ratios were used to assess treatment effects and pairwise differences between treatment means were assessed using t-tests.

Results

Microclimatic conditions

In the first week of Experiment 1 there were five wetness periods of necrotic lily leaves recorded and four

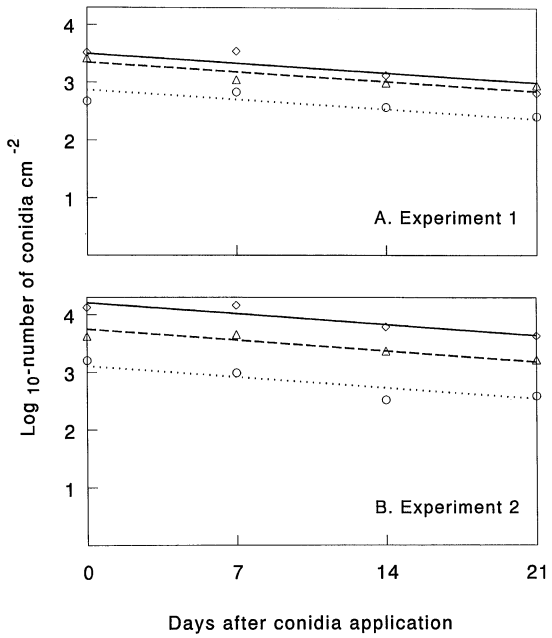

Figure 1. Number of conidia of *Ulocladium atrum* on leaves at the top (-◇-), middle (-△-) and bottom (···○···) level of the lily canopy. The regression equation was $\log_{10}$ (number of conidia cm^{-2}) = a + b(time). Intercept values (a) were 3.50, 3.35, and 2.87 (LSD = 0.2945; $P < 0.05$) for leaves located at the top, middle and bottom canopy levels (Experiment 1) and were 4.21, 3.75, and 3.11 (LSD = 0.2250; $P < 0.05$) for leaves located at the top, middle and bottom canopy levels in Experiment 2. Slope (b) values were -0.0238 (Standard error = 0.005) and -0.0271 (Standard error = 0.005) for Experiment 1 and Experiment 2, respectively.

of these were longer than 10 h duration (Table 1). In contrast, leaf wetness periods were relatively short and only one rain event was recorded in the first week of Experiment 2 (Week 2, Table 1). The following weeks were characterised by relatively short leaf wetness periods. The only significant rain event (rainfall > 5 mm) was recorded on 27 July. Several minor rain events ($\leq$ 0.5 mm) were also recorded but these did not result in leaf wetness inside the dense lily canopy. During leaf wetness periods, temperatures ranged between 7.2 °C and 24.8 °C with an average of 14.3 °C. Temperatures above 25 °C consistently occurred during the five weeks of experimentation.

Conidia density and germination on green lily leaves

Conidia density

The background number of *U. atrum* conidia on green leaves at each level in the lily canopy in the water treated control plots was very low (< 60 cm^{-2}) at all sampling times in Experiment 1 and less than

Table 1. Microclimate measurements during the field experimentation (Wageningen, 24 July - 4 September 1995)

Week number[a]	Wetness periods of necrotic lily leaves			Rain events		Average temperature (°C) recorded during;	
	Total number	Average duration (h)	Total duration (h)	Total number	Total amount (mm)	The whole period[b]	Leaf wetness periods[b]
1	5	17.1	85.5	5	11.0	20.5 (13.0–29.8)	17.9 (13.0–24.8)
2	5	6.7	33.5	1	0.2	21.4 (12.0–30.3)	14.8 (12.0–19.3)
3	5	5.0	25.0	1	0.2	19.3 (8.5–30.5)	12.7 (8.5–18.8)
4	7	6.9	48.0	4	0.8	19.3 (7.2–30.1)	13.0 (7.2–19.8)
5	9	7.1	64.0	12	6.0	18.3 (9.0–29.7)	14.4 (9.0–19.0)
6	7	3.1	22.0	12	7.4	13.5 (5.4–20.2)	12.9 (11.3–16.7)

[a] Experiment 1 and 2 commenced on 24 and 31 July, respectively.
[b] Values are the average temperature and figures in parenthesis are the range.

160 cm^{-2} at all sampling times in Experiment 2. The number of conidia on lily leaves sampled directly after *U. atrum* application was 4184, 2664 and 816 cm^{-2} for leaves at the top, middle and bottom canopy levels, respectively, in Experiment 1 (Figure 1a). Significantly ($P < 0.01$) less conidia were found on leaves from the bottom of the canopy compared to the middle or top levels of the canopy. In Experiment 2, the number of conidia on lily leaves sampled directly after spraying was 14040, 4720 and 2464 cm^{-2} for leaves at the top, middle and bottom levels of the canopy, respectively. In this experiment, the number of *U. atrum* conidia cm^{-2} at each leaf level was significantly different ($P < 0.05$). The number of *U. atrum* conidia cm^{-2} on lily leaves declined over time in both experiments and after 21 days there were 672, 1264 and 336 conidia cm^{-2} on leaves at the top, middle and bottom level of the lily canopy, respectively (Experiment 1). In Experiment 2, the number of *U. atrum* conidia cm^{-2} declined to 5112, 1920 and 512 on leaves at the top, middle and bottom levels of the lily canopy, respectively. There were significant ($P < 0.01$) effects of canopy level and linear time ($P < 0.001$) based on the calculated P-values of the F-tests in both experiments. The decline of number of conidia cm^{-2} over time was best described by a linear polynomial with different intercepts for canopy levels. Since there were no significant ($P > 0.05$) differences in the rate of decline of conidia density on leaves at each canopy level, a single slope value (b) is presented (Figure 1). Despite large differences in the initial density of conidia on green leaf surfaces in Experiment 1 and 2, the rate of decline in both experiments was similar and was not significantly ($P > 0.05$) different.

Field germination and germination potential

Seven days after *U. atrum* application, the germination of conidia on green leaf surfaces in the field (mean of all leaf levels) was 81% and 60% in Experiment 1, and Experiment 2, respectively (Table 2). There were no significant ($P > 0.05$) increases in field germination after seven days in both experiments. Field germination was significantly ($P < 0.05$) lower on leaves sampled from the top level of the canopy, compared to the middle or bottom levels in Experiment 1. In contrast, canopy level had no significant ($P > 0.05$) effect on field germination in Experiment 2. In both experiments there were no significant ($P > 0.05$) time and canopy level interactions.

At the time of *U. atrum* application, germination potential was 100% in both experiments. Germination potential was significantly ($P < 0.05$) greater than field germination at all sampling times in Experiment 1 (Table 2). Seven days after application, germination potential declined to 88% (mean of three canopy levels) but thereafter there were no significant ($P > 0.05$) changes over time. Germination potential of conidia on leaves at the bottom level of the canopy was significantly ($P < 0.05$) higher than conidia deposited on the top level of the canopy at all sampling times. In Experiment 2, germination potential (mean of all canopy levels) was 26% higher than the percentage field germination at the seven day assessment. In Experiment 2, germination potential declined significantly ($P < 0.01$) from 86% (mean of all canopy levels) to 61% at Day 21 and in contrast to Experiment 1, there were no significant ($P > 0.05$) canopy level effects.

Table 2. Germination of *Ulocladium atrum* conidia on green lily leaves (cv. Mont Blanc) at different canopy levels in the field (field germination) and after subsequent incubation at high humidity (germination potential)

Sample time*	Canopy level	Field Germination (%)	Germination potential (%)**
Experiment 1			
Day 7	Top	75 b	83 b
	Middle	83 ab	89 a
	Bottom	85 a	91 ab
Day 14	Top	72 b	80 b
	Middle	85 a	85 ab
	Bottom	76 b	91 a
Day 21	Top	62 b	75 b
	Middle	78 a	76 b
	Bottom	71 ab	90 a
Experiment 2			
Day 7	Top	56 a	86 a
	Middle	62 a	79 a
	Bottom	62 a	93 a
Day 14	Top	58 a	67 a
	Middle	58 a	74 a
	Bottom	47 a	72 a
Day 21	Top	43 a	64 a
	Middle	54 a	56 a
	Bottom	56 a	62 a

* Time after application of *U. atrum*.
** Percent germination after sampling and additional incubation in high humidity chambers at 18 °C in the dark for 18 h. Values within the same column and each sampling time with a common letter do not differ significantly (LSD-test of angular-transformed values; $P < 0.05$).
There were no significant ($P > 0.05$) differences detected for percentage germination between different sample times and canopy level variables.

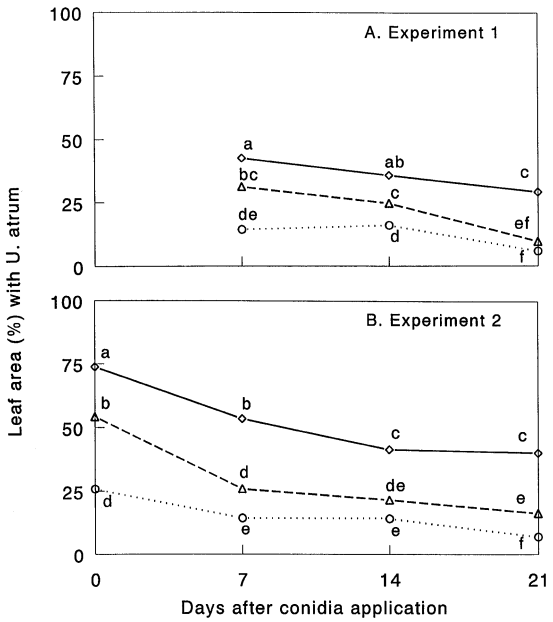

Figure 2. Colonisation of necrotic lily leaves by *Ulocladium atrum* sampled from the top (-◇-), middle (-△-) and bottom (···○···) levels of the canopy. Leaves were sprayed with *U. atrum* at Day 0. Necrosis was induced at Day 0, 7, 14 and 21. Leaves were sampled 14 days after induction of necrosis, incubated for 9 days at 18 °C in moist chambers and the leaf area covered with conidiophores of *U. atrum* was estimated. Values with a common letter do not differ significantly at $P < 0.05$ (t-test of angular-transformed values).

Colonisation of artificially induced necrotic lily leaves by U. atrum

The ability of surviving *U. atrum* inoculum on green leaf surfaces to successfully colonise necrotic tissues was measured after paraquat induction of necrosis on tagged green leaves. Necrosis induction was not successful immediately after *U. atrum* application (Day 0) in Experiment 1 and *U. atrum* colonisation of necrotic tissue was not recorded. On leaves in the control treatment without *U. atrum* application, the leaf area colonised by *Ulocladium* spp. was 1.6% and 4.4% in Experiment 1 and Experiment 2, respectively. Up to 74% of the necrotic leaf area was colonised by *U. atrum* after the leaves were treated with this antagonist (Figure 2). Leaf area colonisation by *U. atrum* was always highest on the leaves from the top level of the canopy and lowest for leaves sampled from the bottom canopy level in both experiments. This pattern was consistent for all times of necrosis induction and there was a significant ($P < 0.05$) effect of canopy level on *U. atrum* colonisation.

The colonisation of necrotic leaves by *U. atrum* decreased over time. When necrosis was induced immediately after antagonist application (Day 0), colonisation of the leaf area by *U. atrum* was 74%, 54% and 26% for leaves sampled from the top, middle and bottom canopy levels, respectively (Figure 2b, Experiment 2). In contrast, when necrosis was induced 21 days after application of the antagonist, colonisation was 40%, 16% and 7% for the three canopy levels, respectively. Seven days after antagonist application (Day 7, Figure 2a), leaf area colonisation by *U. atrum* was 43%, 31% and 14% for leaves sampled from the top, middle and bottom canopy levels, respectively, in Experiment 1. When necrosis was induced 21 days after application of the antagonist (Day 21, Figure 2a), colonisation was 29%, 10% and 6% for the top middle and bottom canopy levels, respectively. Despite a

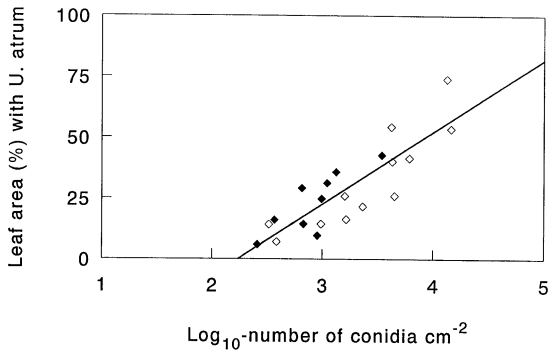

Figure 3. Relationship between density of *Ulocladium atrum* conidia on the green leaf surface of Asian lilies (cv. Mount Blanc) prior to induction of necrosis and leaf area (%) covered with conidiophores of *U. atrum*. Number of conidia cm^{-2} of leaf tissue was log-transformed. Regression equation is of the form % leaf area with *U. atrum* = −66.23 +29.61 (log_{10} number of conidia cm^{-2}); R^2 = 0.713; $P < 0.001$). Data from Experiment 1 (♦) and Experiment 2 (◊) were pooled for analysis.

reduction of *U. atrum* colonisation 21 days after antagonist application colonisation was still greater than in untreated control treatments (17%, 1% and 1%, Experiment 1 and 2%, 2% and 1%, Experiment 2), for the top middle and bottom levels, respectively.

For both experiments, ANOVA indicated that there were significant ($P < 0.05$) linear and quadratic effects over time. A quadratic polynomial in time with different intercepts and slopes for the linear effect was the best model which fitted the data for both experiments. However, pairwise comparisons within canopy levels indicated that the decrease of colonisation by *U. atrum* in time was not significant ($P < 0.05$) in all cases (Figure 2).

U. atrum *conidial density and colonisation relationship*

For *U. atrum* treated leaves, there was a significant ($P < 0.001$) relationship between the density of *U. atrum* conidia (germinated and ungerminated) assessed on leaves prior to induction of necrosis and the leaf area (%) covered in *U. atrum* found after induction of necrosis, field exposure for a further 15 days, and subsequent incubation in moist chambers in the laboratory (Figure 3).

Colonisation of artificially induced necrotic lily leaves by commonly occurring saprophytic fungi

The dominant saprophytic fungi detected on lily leaves after artificial induction of necrosis were *Alternaria* spp. and *Cladosporium* spp. Several other saprophytic fungi such as *Epicoccum* spp., *Stemphylium* spp. and *Gonatobotrys* spp. covered only 1% or less of the area of necrotic leaves (mean of all leaves) in the untreated water control plots. *Botrytis* spp. were found sporadically and only on three and five leaves throughout the entire periods of Experiment 1 and 2, respectively. The leaf area covered with conidiophores of *Botrytis* spp. for all leaves in the untreated plots was less than 0.05% in both experiments. The effect of *U. atrum* application to green lily leaves on subsequent colonisation of leaf tissue by common saprophytic fungi after induction of necrosis was investigated for the *Alternaria* spp. and *Cladosporium* spp. The occurrence of other saprophytic fungi was sporadic and was therefore not analysed further.

Alternaria *spp.*

In the absence of *U. atrum*, and after incubation in high humidity chambers, *Alternaria* spp. sporulation on necrotic leaves was 16.3% (Experiment 1) and 18.4% (Experiment 2) of the leaf area when averaged over all times of necrosis induction and canopy level. Leaves sampled from the bottom canopy level were generally less colonised by *Alternaria* spp. and the occurrence of *Alternaria* spp. tended to increase with time (Table 3).

There were significant ($P < 0.01$) treatment and leaf level interactions detected (at each necrosis induction time) and the statistical comparisons are summarised for each canopy level at each necrosis induction time. The application of *U. atrum* resulted in significantly ($P < 0.05$) reduced colonisation of lily leaves by *Alternaria* spp. at all times of necrosis induction on leaves from the top and the middle canopy levels and in two out of the seven observations on leaves from the bottom level. *Alternaria* spp. sporulation was reduced by between 63 and 78% in Experiment 1 and by 51 and 90% in Experiment 2 on leaves from the top and middle canopy levels. *U. atrum* suppression of *Alternaria* spp. declined over time in Experiment 2 (90% at Day 0 and 66% at Day 21). This decline was statistically not further analyzed since there was evidence that *Alternaria* spp. colonisation in the control treatment increased over time (16% at Day 0 to 31% at Day 21, Table 3).

Cladosporium *spp.*

In the absence of *U. atrum*, and after incubation in high humidity chambers, *Cladosporium* spp. sporulation on necrotic leaves was 7.4% (Experiment 1) and

Table 3. Effect of *Ulocladium atrum* application on colonisation of artificially induced necrotic lily leaf tissue by *Alternaria* spp.

Treatment	Canopy level	Percentage leaf area covered with conidiophores of *Alternaria* spp.[a]			
		0 days[b]	7 days	14 days	21 days
Experiment 1					
Water	Top	–[c]	10.5 *	18.0 *	27.7 *
U. atrum		–	3.8 (64)	5.3 (71)	6.0 (78)
Water	Middle	–	12.8 *	20.1 *	24.8 *
U. atrum		–	4.8 (63)	6.5 (68)	8.4 (66)
Water	Bottom	–	6.1 n.s.	10.3 n.s.	16.4 *
U. atrum		–	5.2 (15)	6.7 (35)	8.2 (50)
Experiment 2					
Water	Top	16.1 *	17.8 *	29.4 *	30.5 *
U. atrum		1.6 (90)	2.9 (84)	7.0 (76)	10.5 (66)
Water	Middle	20.8 *	19.4 *	19.4 *	20.2 *
U. atrum		3.2 (85)	5.7 (71)	9.4 (52)	10.0 (51)
Water	Bottom	10.4 n.s.	11.5 n.s.	15.8 *	10.0 n.s.
U. atrum		6.3 (39)	5.9 (49)	6.6 (58)	7.3 (27)

[a] Assessed after leaf samples were incubated in high humidity chambers at 18 ° C in the dark for 9 days.
[b] Necrosis was induced 0, 7, 14 and 21 days after application of *U. atrum* onto green leaves.
[c] Not assessed.
* Significantly different at $P < 0.05$ from *U. atrum* treatment (t-test of angular-transformed values).
n.s. = no significant difference.
Figures in parentheses are percent reduction of *Alternaria* spp. sporulation in comparison to the water treatment.

12.0% (Experiment 2) of the leaf area when averaged over all times of necrosis induction and canopy level. In contrast to *Alternaria* spp., there did not appear to be any trend of *Cladosporium* spp. colonisation over time. The application of *U. atrum* resulted in significantly ($P < 0.05$) reduced colonisation of *Cladosporium* spp. on lily leaves by up to 97% when necrosis was induced on the same day as the *U. atrum* application (Table 4). The suppression of *Cladosporium* spp. by *U. atrum* tended to decrease over time and was generally lower on leaves at the bottom canopy level. When necrosis was induced 21 days after *U. atrum* application, there were no significant ($P > 0.05$) reductions of *Cladosporium* spp. sporulation in both experiments.

Discussion

Conidia of an antagonist deposited on aerial plant surfaces are exposed to large and rapid changes in temperature, water availability and radiation (Burrage, 1971). In addition to surviving these extremes, effective antagonists must have the capacity to grow rapidly during the limited periods of leaf wetness that are favourable for growth and be able to colonise the host substrate. Successful colonisation of the host also depends on sufficient viable antagonist inoculum to outcompete pathogen inoculum and commonly occurring saprophytes.

The fungal antagonist *U. atrum* has reduced *B. cinerea* colonisation of necrotic tissues of lily consistently by 90% in field studies (Köhl et al., 1995b). When exposed to field conditions *U. atrum* survived and also colonised necrotic kiwifruit leaves (Elmer et al.,1995) in the presence of *Botrytis* spp. and other competing saprophytes. The apparent success of this antagonist may be attributed to several factors. The dark pigment, melanin, present in black walled dermatiaceous spores (Diem, 1971), including *U. atrum*, may provide protection against UV radiation. Germination of *U. atrum* conidia on dead lily tissue was reported to occur in a short time frame with up to 20% germination after only four h in the field and greater than 90% germination after leaf wetness periods of 18 h (Köhl et al., 1995b). *U. atrum* survived for several days on necrotic tissues in the field without any loss of germination capacity and indicated that *U. atrum* conidia were not affected by microclimate conditions during that time. In order to gain a greater understanding of the behaviour of *U. atrum* in the phyllosphere, we obtained information on the density, survival and

Table 4. Effect of *Ulocladium atrum* application on colonisation of artificially induced necrotic lily leaf tissue by *Cladosporium* spp.

Treatment	Canopy level	Percentage leaf area covered with conidiophores of *Cladosporium* spp.[a]			
		0 days[b]	7 days	14 days	21 days
Experiment 1					
Water	Top	–[c]	5.2 *	5.4 *	10.4 n.s.
U. atrum		–	0.8 (85)	2.0 (63)	4.6 (56)
Water	Middle	–	6.1 n.s.	5.5 *	9.3 n.s.
U. atrum		–	3.8 (38)	2.7 (51)	11.5 (−24)
Water	Bottom	–	7.3 n.s.	6.7 *	10.8 n.s.
U. atrum		–	4.9 (33)	3.3 (51)	11.9 (−10)
Experiment 2					
Water	Top	9.8 *	8.7 *	12.7 *	13.7 n.s.
U. atrum		0.3 (97)	1.0 (89)	2.8 (78)	10.2 (26)
Water	Middle	8.1 *	7.9 *	10.1 *	21.9 n.s.
U. atrum		3.6 (56)	2.7 (66)	5.5 (46)	21.2 (3)
Water	Bottom	11.5 *	7.9 n.s.	10.1 n.s.	21.7 n.s.
U. atrum		4.1 (64)	6.6 (16)	11.2 (−11)	19.2 (12)

[a] Assessed after leaf samples were incubated in high humidity chambers at 18 ° C in the dark for 9 days.
[b] Necrosis was induced 0, 7, 14 and 21 days after application of *U. atrum* onto green leaves.
[c] Not assessed.
* Significantly different at $P < 0.05$ from *U. atrum* treatment (t-test of angular-transformed values).
n.s. = no significant difference.
Figures in parentheses are percent reduction of *Cladosporium* spp. sporulation in comparison to the water treatment.

colonisation ability of *U. atrum* conidia on the green leaf surfaces of Asian lilies in the field as influenced by (1) the vertical nature of the lily canopy and (2) the duration of exposure to field conditions.

The number of *U. atrum* conidia cm^{-2} on green leaves at the top and middle canopy levels was not significantly different (Experiment 1), immediately after *U. atrum* application to lily microplots with a propane powered backpack. However, conidia density was significantly less on green leaves at the lower canopy level compared to the middle and top levels. In the second experiment, the number of conidia was significantly different at each level of the canopy. In both experiments the number of conidia detected on leaves at the bottom level of the canopy was 20% (Experiment 1) and 18% (Experiment 2) of that which was deposited onto leaves at the top of the lily canopy. An uneven vertical distribution of antagonist inoculum may have significant epidemiological consequences since primary infections in host canopies may commence on the older leaves at the bottom of the canopy where host and microclimate conditions are likely to be more conducive to infection and sporulation. These results indicate that the spraying system used in these studies resulted in uneven vertical distribution of antagonist inoculum in the lily canopy and that further evaluation of application methods may be required to improve canopy penetration of the antagonist suspension.

After antagonist application, the number of *U. atrum* conidia on green leaf surfaces declined at a similar rate at each canopy level in both experiments (Figure 1; Table 1). This result is in contrast to earlier findings in 1994 which indicated that a loss of *U. atrum* conidia from the top of the lily canopy coincided with an accumulation of conidia on the bottom leaves of the canopy (Köhl et al., unpublished). The 1994 growing season was characterised by more frequent rain events compared to 1995 and indicates that rain splash may be an important mechanism for redistribution of antagonist inoculum. Rain splash is an efficient mechanism for spreading large numbers of pathogen propagules from a source of inoculum but the relative role of rain and wind dispersal has been described only for some pathogens (Fitt et al., 1989; Vloutoglou et al., 1995). The relative importance of rain versus wind as a dispersal mechanism for redistributing biological antagonists such as *U. atrum* is poorly understood. The use of molecular markers may enable better investigation of the spatial and temporal dynamics of *U. atrum* as was recently reported for

the antagonist *Trichoderma* harzianum (Bowen et al., 1996).

The number of *U. atrum* conidia on green lily leaf surfaces after 21 days exposure to field conditions declined by 70% and 73% in Experiment 1 and 2, respectively. Only one significant rain event was recorded over the duration of this experimentation and suggests that other factors may also be responsible for the displacement of conidia. The use of stickers in fungicide formulations is common and has improved chemical persistence on plant surfaces. The formulation of *U. atrum* used in these studies was a spore suspension in water with Tween 80 and it is likely that improved adhesion of antagonist spores could be achieved by improving the formulation of the antagonist suspension.

Germination of *U. atrum* conidia on green leaves in the field reached a maximum of 81% and 60% seven days after antagonist application in Experiment 1 and 2. Field germination was lower in Experiment 2 compared to Experiment 1. Köhl et al. (1995b) suggested that germination on necrotic leaf surfaces was dependant upon the duration of leaf wetness and temperatures above 10 °C. Low temperatures during the wetness periods were reported to hamper germination. In the first seven days of Experiment 1, five leaf wetness periods with an average duration of 17.1 h were recorded. The average temperature during the leaf wetness periods was 17.9 °C. In contrast, the average duration of leaf wetness in the first seven days of Experiment 2 was 6.7 h with an average temperature of 14.8 °C. Shorter leaf wetness durations may have been the factor responsible for a reduction of conidia germination on green lily leaf surfaces.

Long term survival on green leaf surfaces is a necessary attribute for many successful saprophytic species since spores which land on the leaf surface must wait for the onset of senescence in order to penetrate the host tissue. Conidial viability (germination potential) declined slightly (100% to 88%) after seven days exposure to field conditions (mean of three canopy levels, Experiment 1). Thereafter, there were no significant changes in germination potential even after 21 days. Germination potential was significantly higher on leaves from the bottom level of the lily canopy (Experiment 1), but germination potential was not affected by canopy level in Experiment 2. It is possible that direct exposure to UV radiation may increase conidial mortality and host tissue shading may provide some protection. Spore survival in the field was also reported to be greater on the underside of leaves compared to upper leaf surfaces (Ceasar and Pearson, 1983; Rotem et al., 1985). This suggests that there is a need to study further *U. atrum* survival on the underside of leaves at different canopy levels.

The success of a potential antagonist is dependent not only upon antagonist inoculum remaining viable for prolonged periods of time, but also on retention of cellular functions required for rapid colonisation of the host substrate. In our study, we found a significant decline in the capacity of germ tubes to produce new extension growth, indicating a loss of germling vigour, when *U. atrum* conidia were incubated in high humidity chamber after an exposure to field conditions for 21 days on green leaves (Data not presented). In addition, the number of new germ tubes arising from conidia exposed in the field for 21 days was also significantly reduced. A reduction of germ tube development may affect the ability of *U. atrum* to remain competitive on the phyllosphere and subsequently colonise host tissue at the onset of senescence.

In our study the ability of surviving *U. atrum* inoculum on green leaf surfaces to successfully colonise necrotic tissues was measured after paraquat induction of necrosis on tagged green leaves. Colonisation by *U. atrum* was consistently highest on necrotic leaves at the top level of the canopy and consistently lower on the necrotic leaves sampled from the bottom canopy level in both experiments. This pattern was also consistent for all times of necrosis induction and indicates that the density of *U. atrum* conidia on the green leaf surface prior to artificial induction of necrosis had a profound effect on the level of necrotic lily leaf colonisation. Necrotic leaf colonisation by *U. atrum* decreased over time (Figure 2a and b) from 51% (averaged over the three canopy levels) when necrosis was induced immediately after antagonist application (Day 0) to 21% (averaged over the three canopy levels) when necrosis was induced 21 days after antagonist application (Experiment 2). A significant decline in colonisation may have been due to a combination of factors. Conidial density declined significantly over time and regression analysis indicated a significant relationship between *U. atrum* density on green leaves, prior to artificial induction of necrosis, and subsequent *U. atrum* colonisation of necrotic lily tissue. A reduction in germination potential, germ tube elongation and number of germ tubes per conidium may have also contributed to a decline in colonisation after 21 days in the field.

The ability of *U. atrum* inoculum to compete with common saprophytic species on artifically induced

necrotic tissues was measured. When necrosis was induced immediately after antagonist application (Table 3, Day 0, Experiment 2), *U. atrum* completely colonised necrotic lily leaves and outcompeted commonly occurring saprophytic *Alternaria* spp. and *Cladosporium* spp. The ability of *U. atrum* to significantly reduce colonisation by *Alternaria* spp. and *Cladosporium* spp. was maintained throughout the 14 days (*Cladosporium* spp.) and 21 days (*Alternaria* spp.) of each experiment. The reduction of *Alternaria* spp. colonisation on necrotic tissues was generally less on leaves at the bottom level of the canopy. Lower numbers of *U. atrum* conidia reaching the bottom leaves of the canopy may account for a reduction of competitive ability since basic ecological principles state that the effects of competition are density dependant (Begon et al., 1986). Our results suggested that when the density of *U. atrum* was relatively low (approximately 1000 conidia cm^{-2}), the reduction of *Alternaria* spp. colonisation compared to the water treatment was not significant. This is potentially important information and indicates that high densities of *U. atrum* conidia (approximately greater than 4000 cm^{-2}) on green leaves should be maintained to ensure that colonisation by naturally occurring saprophytes is effectively suppressed. Such high numbers are practical and were achieved immediately after field application to the top leaves of the canopy (Experiment 1) and the top and middle leaves in Experiment 2. This information is required in order to optimise *U. atrum* application methodology.

In our study we found that *U. atrum* had the ability to survive and persist in the lily phyllosphere for up to 21 days in the field. In addition, *U. atrum* colonised artificially induced necrotic tissues and competed successfully against naturally occurring saprophytes on Asian lily leaves in The Netherlands. Although *Botrytis* spp. were not present in our experimental plots in 1995, these studies have added substantially to our knowledge of the behaviour of *U. atrum* in the field and provides further evidence that *U. atrum* has the necessary characteristics to be a successful biological control agent of *Botrytis* spp. on several host crops.

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