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Effect of interrupted leaf wetness periods on suppression of sporulation of *Botrytis allii* and *B. cinerea* by antagonists on dead onion leaves

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Abstract

Saprophytic antagonists were evaluated for suppression of sporulation of *Botrytis allii* and *B. cinerea* on artificially killed segments of onion leaves that were pre-inoculated with the pathogens. During incubation of the antagonist-treated leaf segments in moist chambers, periods of leaf wetness and leaf dryness were alternated to simulate conditions in the field. Interruption of humid conditions with dry periods had a differential effect on antagonists. *Alternaria alternata, Chaetomium globosum, Ulocladium atrum* and *U. chartarum* suppressed sporulation of *B. allii* almost completely under continuously wet conditions, and when the leaf wetness periods were interrupted with drying periods of 9h imposed 16, 40, and 64 h after the antagonists were applied. When leaf wetness was interrupted 16 h after antagonist application, the number of conidia of *B. allii* produced cm^{-2} leaf surface after eight days was under the detection limit of 5.2×10^3 conidia on leaves treated with these antagonists compared to 3.7×10^5 conidia on leaves that were not treated. On the other hand, *Gliocladium roseum, G. catenulatum* and *Sesquicillium candelabrum*, all highly efficient under continuously wet conditions, were of low to moderate efficiency when leaf wetness periods had been interrupted 16 h after application of the antagonists. The antagonists showed the same differentiation and sensitivity to interrupted wetness periods when tested with *B. cinerea*.

Introduction

Botrytis spp. cause destructive diseases on above-ground parts of plants and harvested products in many different kinds of crops [Maude, 1980]. Until now, disease control is highly dependent on frequent use of fungicides. In view of the threat of rapid build up of fungicide resistance of *Botrytis* spp. [Gullino, 1992] and of environmental concerns over fungicide use, there is an urgent need for alternative control strategies such as biological control. Suppression of sporulation of necrotrophic pathogens such as *Botrytis* spp. is a valid strategy to slow down the progression of polycyclic epidemics caused by these pathogens as shown by Köhl *et al.* [1995] for *B. cinerea* in onions. Saprophytic antagonists may compete with saprophytically growing *Botrytis* spp. in necrotic tissue of the host plant resulting in a suppression of sporulation of the pathogen. The long interaction time between antagonist and pathogen during colonization of necrotic tissue makes this strategy attractive compared to a strategy aimed at prevention of infection: germinating conidia of *Botrytis* spp. penetrate host tissue within a few hours, escaping from antagonists established in the phyllosphere [Fokkema, 1993; Köhl and Fokkema, 1994].

Control of necrotrophic leaf pathogens by antagonists that reduce saprophytic growth of the pathogens in necrotic tissue was demonstrated by Newhook [1957] for *B. cinerea* in tomato, by Cook [1970] for *Fusarium roseum* f. sp. *cerealis* 'Culmorum' in wheat and by Boland and Hunter [1988] and Zhou and Reeleder [1991] for *Sclerotinia sclerotiorum* in snap beans. Antagonists were found also to successfully suppress conidiation of *Cochliobolus sativus* on necrotic leaf spots in wheat [Biles and Hills, 1988] and of *B. cinerea* on strawberry leaves [Sutton and Peng, 1993], as well as ascospore production of *Pyrenophora tritici-repentis* in wheat leaf debris [Pfender, 1988] and of

Venturia inaequalis on fallen apple leaves [Heye and Andrews, 1983].

The aim of our study was to select saprophytic antagonists that are able to suppress sporulation of *Botrytis* spp. on necrotic leaf tissue. *Botrytis allii* and *B. cinerea*, causing onion neck rot and onion leaf spot, respectively, were chosen as model pathogens. A bioassay based on dead segments of onion leaves pre-inoculated with *B. allii* or *B. cinerea* was developed. During incubation of antagonist-treated leaf segments in moist chambers, leaf wetness periods were interrupted by dry periods to simulate field conditions. The adaptation of antagonists to rapid changes between wet and dry periods in necrotic tissue of above ground plant parts may be a key factor for field performance of antagonists aimed at suppression of sporulation of necrotrophic leaf pathogens.

Materials and methods

Fungi

Botrytis allii Munn (syn. *B. aclada* Fres.) and *B. cinerea* Pers. ex Pers. were isolated from an onion seed and a gerbera flower, respectively. The following fungi (with isolate number) were isolated from necrotic leaf tips of onions grown in a field at our institute: *Alternaria alternata* (Fr.) Keissler (300, 317, 319), *A. infectoria* Simmons (264, 270), *Arthrinium* sp. (anamorph state of *Apiospora montagnei* Sacc.) (242), *A. phaeospermum* (Corda) M. B. Ellis (243, 244), *Aureobasidium pullulans* (de Bary) Arnaud (490), *Chaetomium globosum* Kunze ex Fr. (256), *Cladosporium cladosporioides* (Fres.) de Vries (564), *C. herbarum* (Pers.) Link ex Gray (587, 571, 593), *Penicillium brevicompactum* Dierckx (221), *P. hirsutum* Dierkx (211), *P. spinulosum* Thom (201), *Penicillium* spp. (023, 025), *Sesquicillium candelabrum* (Bonord.) W. Gams (249), *Ulocladium atrum* Preuss (385), *U. chartarum* (Preuss) Simmons (380), *Verticillium nigrescens* Pethybr. (250). *Gliocladium catenulatum* Gil. & Abbott (1814), *G. nigrovirens* Van Beyma (1815) and *G. roseum* Bain. (1813), were isolated from peel of potato tubers. *Trichothecium roseum* (Pers.) Link ex Gray (706) originated from a necrotic leaf of lily, *Cryptococcus luteus* (Saito) Skinner (WCS36) from a rye leaf. *Trichoderma hamatum* (Bon.) Bain aggr. (003, T166), *T. harzianum* Rifai aggr. (T000, 022, T154) and *T. viride* Pers. ex S.F. Gray aggr. (T004, T048, T122, T141, T218, T226) were isolated from soil or bark [Köhl and Schlösser, 1988]. *G. catenulatum* (162) and *G. roseum* (160, 161), isolated from roots of red clover, were kindly provided by P. Lüth, Prophyta Biologischer Pflanzenschutz GmbH, Malchow, Germany. *T. harzianum* 39, isolated from a cucumber fruit grown in a glasshouse, was kindly provided by Y. Elad, Volcani Center, Bet Dagan, Israel.

Leaf segments

Symptomless green leaves were removed from 12-week-old field-grown plants of onion cv Hyton, dried for several days at 60 °C and stored in paper bags at room temperature in the dark. Dry leaves were cut into segments each 2 cm long, sealed in plastic bags and sterilized by gamma radiation of 4 Mrad.

Conidial suspensions

All fungi were cultured in petri dishes on oat meal agar (20 g oatmeal, 15 g agar, 1000 ml tap water) except *A. pullulans* 490 and *C. luteus* WCS36 which were grown on basal yeast agar (10 g bacteriological peptone, 20 g sucrose, 1 g yeast extract, 20 g agar, 1000 ml tap water). Cultures of all fungi were incubated at 18 °C in the dark for 10–14 days, except those of *A. pullulans* and *C. luteus*, which were cultured for three days, and of *Alternaria* spp. and *Ulocladium* spp., which were cultured for 21 days. To obtain spore suspensions, cultures were flooded with sterile tap water containing 0.01% Tween 80. After gently rubbing with a rubber spatula to remove spores from fungal cultures, suspensions were filtered through a sterile nylon gauze with a mesh of 200 μm. Concentrations of conidial suspensions were determined with the aid of a haemocytometer and adjusted with sterile tap water containing 0.01% Tween 80 to $1x10^6$ spores ml^{-1} for antagonists, $1x10^4$ or $1x10^5$ conidia ml^{-1} for *B. allii* and $1x10^3$, $1x10^4$ or $1x10^5$ conidia ml^{-1} for *B. cinerea*.

Bioassay

Onion leaf segments for use in bioassays were washed thoroughly with tap water to remove soluble nutrients. Approximately 100 segments were washed three times in 350 ml aliquots of sterile tap water contained in sterile 500 ml conical flasks which were shaken at 150 strokes $min.^{-1}$ for 30 min. The segments were subsequently blotted dry with sterile filter paper. Four leaf segments were placed in each of a series of moist chambers. Each chamber consisted of a sterile plastic petri dish (90 mm diameter) containing two sterile filter

papers (80 mm diameter) and 1 ml sterile tap water. Experiments were carried out in a completely randomized design with five replications (petri dish) for treatments with antagonists and five or ten replications for the control.

Conidial suspensions of *B. allii* or *B. cinerea* were sprayed on the leaf segments in the moist chambers and the segments were incubated at 18 °C for 24 h (*B. allii*) or 8 h (*B. cinerea*). Immediately after the incubation periods, spore suspensions of antagonists, or water plus surfactant, were sprayed on the segments. The pathogens and antagonists were applied with sterile atomizers at approximately 5 μl cm^{-2} leaf segment. Thereafter, leaves were further incubated at 18 °C in the dark in continuous wetness periods or in wetness periods that were interrupted 16, 40, or 64 h after the antagonist were applied. To interrupt wetness periods, leaf segments were placed on two layers of sterile dry filter paper in open petri dishes in laminar flow cabinets for 9 h. Temperatures in the cabinets were approximately 20 °C and relative humidity ranged between 30 and 40 % RH. After the dry period, the filter paper in each petri dish was wetted with 2 ml sterile tap water, and the dishes were closed and kept at 18 °C in the dark. After a total incubation period of eight days, the leaf area covered with conidiophores of *B. allii* or *B. cinerea* was estimated using classes from zero to five, that represented, respectively, 0%, 1–5%, >5–25%, >25–50%, >50–75%, and >75–100% of the leaf surface covered with conidiophores of the pathogens. From the number of leaf segments of each class (n_0–n_5) a sporulation index (SI) ranging from zero to 100 was calculated for each replication (petri dish) consisting of four leaf segments ($SI = (0 \times n_0 + 5 \times n_1 + 25 \times n_2 + 50 \times n_3 + 75 \times n_4 + 100 \times n_5) / 4$).

In total, 41 fungal isolates were tested in bioassays with *B. allii* under continuously wet conditions. Fourteen isolates that had shown strong antagonism in these screening experiments were tested in bioassays (experiments 1–5) with an interrupted wetness period. In experiment 5, the effect of antagonists on sporulation of *B. allii* was also quantified by counting conidia of *B. allii* produced on dead onion leaves. The antagonists *A. alternata* 317 and 319, *A. pullulans* 490, *C. globosum* 256, *G. catenulatum* 162, *G. roseum* 1813, and *U. atrum* 385 were also tested in bioassays on dead leaf segments of onion that were pre-inoculated with *B. cinerea*.

Number of conidia produced in bioassay

Conidial production of *B. allii* on the four leaf segments of each of three arbitrarily chosen replications of each treatment was determined in experiment 5. Leaf surface area was measured using an interactive digitizer (Minimop, Kontron, Oberkochen, Germany). Leaves were homogenized in 5 ml of tap water using a planetary micro mill (Pulverisette 7, Fritsch GmbH Laborgerätebau, Idar-Oberstein, Germany) and water was added to obtain 10 ml suspension. The number of conidia of *B. allii* in four 6.4 μl aliquots of each of the suspensions was counted with the aid of a haemocytometer and the number of conidia of *B. allii* recovered cm^{-2} leaf surface was estimated. The detection limit for conidia was approximately 5200 conidia per square centimetre leaf surface as calculated from the average leaf surface of the four leaf segments per replication (petri dish) which had been homogenized, and the volume of the suspension which had been examined.

Drying process of onion leaves during bioassays

The water content of leaf segments was monitored in one of the bioassays with interrupted leaf wetness. Four leaf segments, similarly treated as leaves of the water treatment of bioassays, were placed in ten replications on dry filter paper in the laminar flow cabinet. The segments were weighed at 60 min intervals for 14 h, and after the leaf segments were dried at 105 °C for 6 h, and the water content of the leaf segments was estimated.

The relationship between water content and water potential of dead onion leaves was determined in an additional experiment. Necrotic leaves of field grown onions were cut into 1- cm segments and dried at 105 °C for 24 h. Sterile glass tubes were filled with 500 mg dried leaf segments. Sterile tap water was added to adjust the water content of the tissue in the individual glass tubes to values ranging from 0.0–3.5 g water g^{-1} dry segments. Values of water content increased in steps of 0.25 g water g^{-1} dry segments in the range between 0.0 and 2.0 g water g^{-1} dry segments, and in steps of 0.5 g in the range of 2.0–3.5 g water g^{-1} dry segments. For each individual value, four replications were prepared. Glass tubes were sealed, shaken thoroughly and stored at 20 °C in the dark for 12 days to allow equilibration of water potential of leaf segments within a tube. Thereafter, water potentials of one subsample per tube were measured with a Decagon thermocouple psychrometer (Decagon Devices Inc., Pullman, WA, USA).

Table 1. Comparative suppression of sporulation of *Botrytis allii* by antagonists on dead segments of onion leaves in bioassays under continuously moist conditions

Reduction of sporulation index[a]	First screening[b]	Second screening[c]
Not significant[d]	*Cryptococcus luteus*WCS36	
< 90% [d]	*Gliocladium nigrovirens* 1815	*Aureobasidium pullulans* 490
	Trichoderma hamatum T166	*Cladosporium cladosporioides* 564
	T. harzianum 39, T000, 022, T154	*C. herbarum* 593
	Penicillium sp. 023, 025	*P. hirsutum* 211
		P. spinulosum 201
		Trichothecium roseum 706
		Verticillium nigrescens 250
> 90% [d]	*G. catenulatum* 1814	*Alternaria infectoria* 264, 270
	G. roseum 1813	*A. alternata* 317
	T. hamatum 003	*Arthrinium* sp. 242
	T. viride T004, T048, T122, T141,	*A. phaeospermum* 243, 244
	T218, T226	*Chaetomium globosum* 256
		C. herbarum 571, 587
		G. catenulatum 162
		G. roseum 1813, 160, 161
		P. brevicompactum 221
		Sesquicillium candelabrum 249
		Ulocladium atrum 385

[a] Antagonists were sprayed with 10^6 spores ml^{-1} 24 hours after application of *B. allii*. Leaves were incubated for 8 days at 18 °C. Sporulation of *B. allii* was scored using classes 0–5, representing, respectively, 0%, 1–5%, >5–25%, >25–50%, >50–75%, and >75–100% of the leaf area covered with conidiophores of *B. allii* and a sporulation index (SI) was calculated ($SI = (0 \times n_0 + 5 \times n_1 + 25 \times n_2 + 50 \times n_3 + 75 \times n_4 + 100 \times n_5) / 4$).

[b] *B. allii* sprayed with 10^4 conidia ml^{-1}.

[c] *B. allii* sprayed with 10^5 conidia ml^{-1}.

[d] Compared to control treatment by LSD-test ($p < 0.05$).

Statistics

The statistical package Genstat 5 was used for data processing [Genstat 5 Committee, 1990]. Data on sporulation index (SI) and log-transformed data on number of conidia were analyzed separately for each experiment by analysis of variance (ANOVA) followed by LSD-tests. Treatments causing complete suppression of sporulation (no variance among replications) were excluded from the analysis.

Results

Bioassays with B. allii *under continuously wet conditions*

From 18 isolates tested in a first screening, eight isolates suppressed sporulation of *B. allii* significantly with efficacies of up to 90% (Table 1). All isolates of *T. harzianum* tested belonged to this group of antagonists. *G. catenulatum* 1814, *G. roseum* 1813, *T. hamatum* 003 and all isolates of *T. viride* tested consistently reduced sporulation of *B. allii* by more than 90%. Only the yeast *Cryptococcus luteus* WCS36 caused no significant reduction of the sporulation index (SI) of *B. allii*.

In a second screening, saprophytes isolated from necrotic leaf tips of field grown onions and several isolates of *Gliocladium* spp. were tested (Table 1). Conidial suspensions of *B. allii* contained 1×10^5 conidia ml^{-1} instead of 1×10^4 conidia ml^{-1} as in the initial screening to allow a better differentiation between strong antagonists. All 23 isolates tested significantly suppressed sporulation of *B. allii* in each of the two repetitions of the bioassays. *A. pullulans* 490, *C. cladosporioides* 564, *G. roseum* 248, *P. hirsutum* 211, *P. spinulosum* 201, *Trichothecium roseum* 706 and *V. nigrescens* 250 showed reduced SI for up to 90%. Isolates of *Alternaria* spp., *Arthrinium* spp., *C. globosum*,

Table 2. Effect of antagonists on sporulation of *Botrytis allii* on dead segments of onion leaves after interruption of leaf wetness

Treatment	Sporulation index (SI)[a] for *B. allii* Time of wetness interruption (h after antagonist applied)					
	0	16	40	64	16 and 40	16, 40, and 60
Experiment 1						
Water	91	78	79	78	59	45
Alternaria alternata 319	0	0	0	0	0	0
Arthrinium sp. 242	0	4	0	0	1	7
Chaetomium globosum 256	0	0	0	0	0	0
Gliocladium roseum 1813	0	42	12	0	45	30
Experiment 2						
Water	79	58	69	89	37	45
Aureobasidium pullulans 490	8	1	2	1	1	1
G. catenulatum 162	0	30	18	0	31	27
Sesquicillium candelabrum 249	0	50	34	19	29	43
Ulocladium chartarum 380	0	0	1	0	0	0
Experiment 3						
Water	44	59	54	33	48	56
A. phaeospermum 243	5	15	3	1	20	20
G. roseum 1813	0	42	15	2	33	33
Trichoderma harzianum 39	0	22	7	1	31	24
U. atrum 385	0	0	0	0	0	0
Experiment 4						
Water	37	47	56	45	43	50
A. alternata 300	0	0	0	0	0	0
A. alternata 317	0	0	0	0	0	0
Cladosporium herbarum 571	0	0	0	2	0	0
G. roseum 1813	0	24	11	0	10	21

B. allii had been sprayed with 1×10^5 conidia ml^{-1} 24 h before antagonists were sprayed with 1×10^6 spores ml^{-1}. Leaves were incubated for 8 days at 18 °C.

$LSD_{\%5}$ for experiment 1 to 4 were 16.4, 14.5, 11.5 and 9.8, respectively. Treatment without sporulation of *B. allii* had been excluded from analysis of variance.

[a] See Table 1.

C. herbarum, *P. brevicompactum*, *S. candelabrum* and *U. atrum* consistently suppressed sporulation of *B. allii* by more than 90%. Strongest antagonism was expressed by four out of five isolates of *Gliocladium* spp., which gave a complete inhibition of *B. allii*. Additionally, several isolates were tested in single bioassays (data not presented). Four isolates of *A. alternata* and one of each of *G. roseum*, *U. chartarum* and *U. consortiale* (Thüm.) Simmons suppressed sporulation of *B. allii* by more than 90%, whereas one isolate of *C. herbarum* and two of *U. chartarum* caused reductions of less than 90%.

Bioassays with B. allii *with an interrupted leaf wetness period*

In the five experiments, interruption of the leaf wetness period had no consistent effect on SI of *B. allii* of water control treatments where only *B. allii* had been applied (Tables 2 and 3). When leaf wetness period had been interrupted repeatedly, SI was lower compared to incubation under continuously moist conditions in experiment 1 and 2, but not in experiment 3 and 4.

Dry periods interrupting humid conditions had a differential effect on antagonists. *A. alternata* 300, 317 and 319, *C. globosum* 256, *U. atrum* 385 and *U. chartarum* 380 suppressed sporulation of *B. allii*

Table 3. Effect of antagonists on number of conidia of *Botrytis allii* produced on dead segments of onion leaves incubated in moist chambers under continuously wet conditions or with an interruption of leaf wetness 16 or 64 h after application of antagonists (Experiment 5)

Treatment	Number of conidia of *B. allii* cm^{-2} leaf area Time of wetness interruption (h after antagonists applied)					
	No		16		64	
Water	238100	(55)[a]	373400a[b]	(70)	731100a	(59)
Alternaria alternata 300	n.d.[c]	(0)	n.d.	(0)	n.d.	(0)
A. alternata 317	n.d.	(0)	n.d.	(0)	n.d.	(0)
A. alternata 319	n.d.	(0)	n.d.	(0)	n.d.	(0)
Arthrinium sp. 242	n.d.	(0)	27400bc	(13)	n.d.	(0)
A. phaeospermum 243	<8300[d]	(4)	12500c	(8)	n.d.	(0)
Aureobasidium pullulans 490	n.d.	(0)	n.d.	(1)	n.d.	(0)
Chaetomium globosum 256	n.d.	(0)	<7800[d]	(5)	n.d.	(0)
Cladosporium herbarum 571	n.d.	(0)	58700bc	(23)	<9600[d]	(2)
Gliocladium roseum 1813	n.d.	(0)	118000ab	(24)	n.d.	(0)
G. catenulatum 162	n.d.	(0)	47200bc	(32)	n.d.	(0)
Sesquicillium candelabrum 249	<6000[d]	(0)	27800bc	(30)	31600b	(26)
Trichoderma harzianum 39	n.d.	(2)	<5200[d]	(13)	n.d.	(0)
Ulocladium atrum 385	n.d.	(0)	<5200[d]	(0)	n.d.	(0)
U. chartarum 380	n.d.	(0)	n.d.	(0)	n.d.	(0)

B. allii had been sprayed with 1×10^5 conidia ml^{-1} 24 h before antagonists were sprayed with 1×10^6 spores ml^{-1}. Leaves were incubated for 8 days at 18 °C.

[a] SI (see Table 1); $LSD_{5\%}$ for SI was 11.8.

[b] Numbers of one column followed by the same letter are not significantly different according LSD-test of log-transformed data ($p < 0.05$).

[c] Not detectable at a detection limit of on the average 5200 conidia cm^{-2} (range from 4000 to 8300 conidia cm^{-2} depending on leaf size).

[d] One or two measurements below detection limit.

almost completely under continuously wet conditions and when wetness periods were interrupted 16, 40, or 64 h after application of antagonists. These antagonists were still highly suppressive when wetness periods were interrupted on up to three consecutive days. *A. pullulans* 490 showed high efficacies against *B. allii* when leaf wetness periods had been interrupted. In contrast, *A. pullulans* 490 did not consistently suppress sporulation of *B. allii* by more then 90 % on leaves that were incubated under continuously wet conditions. These results were consistent with results obtained with this antagonist in preliminary bioassays (Table 1).

On the other hand, *G. roseum* 1813, *G. catenulatum* 162 and *S. candelabrum* 249, all highly efficient under continuously wet conditions, were distinctly less efficient when leaf wetness periods were interrupted 16 h after the antagonists were applied. Under these conditions, *G. roseum* 1813, *G. roseum* 162, *T. harzianum* 39 and *S. candelabrum* 249 reduced the SI of *B. allii* by 48%, 51%, 72% and 36% (mean values of two repeated experiments), respectively, compared to the SI value of the control treatment that was sprayed with *B. allii* only and subjected to a dry period after 16 h. Interruption of wetness after 40 h reduced efficacy of the antagonists less than an interruption after 16 h. When wetness was interrupted after 64 h, both isolates of *Gliocladium* spp. completely suppressed sporulation of *B. allii*, as under continuously wet conditions, but the efficacy of *S. candelabrum* 249 was reduced. *Arthrinium* sp. 242 and *A. phaeospermum* 243 also showed a sensitivity to an interrupted leaf wetness period. The effect of an interrupted leaf wetness period on *C. herbarum* 571 and *T. harzianum* 39 were not consistent in two repetitions of the experiment.

More than 2×10^5 conidia of *B. allii* cm^{-1} leaf surface were found on leaves incubated under continuously moist conditions in the control treatment of experiment 5 (Table 3). No conidia of *B. allii* could be detected on leaves treated with antagonists except a few conidia on leaves that had been treated by *A. phaeospermum* 243 or *S. candelabrum* 249. Consid-

Table 4. Effect antagonists on sporulation of *Botrytis cinerea* on dead segments of onion leaves after interruption of leaf wetness

Treatment	Sporulation index (SI) for *B. cinerea*[a]					
	Leaf wetness period not interrupted			Leaf wetness period interrupted [b]		
	10^{3} [c]	10^{4} [c]	10^{5} [c]	10^{3} [c]	10^{4} [c]	10^{5} [c]
Water	63	64	85	55	61	53
Alternaria alternata 317	0	2	32	0	1	26
A. alternata 319	0	1	12	1	3	16
Aureobasidium pullulans 490	0	5	86	0	8	58
Chaetomium globosum 256	0	0	0	3	14	48
Gliocladium catenulatum 162	0	4	42	18	64	57
G. roseum 016	1	5	53	28	43	42
Ulocladium atrum 385	0	1	15	0	0	1

[a] After incubation of leaves for 8 days at 18 °C. SI see Table 1. $LSD_{5\%}$ for SI was 16.6.
[b] 16 h after application of antagonists.
[c] Concentration of conidial suspension of *B. cinerea* (conidia ml^{-1}) sprayed 8 h before antagonists were sprayed with 1 $\times$ 10^{6} spores ml^{-1}.

ering that the detection limit of the assessment was approximately 5200 conidia cm^{-1} leaf surface, all antagonists reduced conidia production by more than 97%. Conidia of *B. allii* were not detected when leaf wetness periods were interrupted 16 h after *A. alternata* 300, 317 and 319, *A. pullulans* 490 and *U. chartarum* 380 were applied. Thus, considering the detection limit, each of these antagonists reduced the number of conidia by more than 98%. After treatments with *C. globosum* 256, *T. harzianum* 39 and *U. atrum* 385 only a few conidia of *B. allii* were found in one or two replications. On leaves treated with *Arthrinium* sp. 242, *A. phaeospermum* 243, *C. herbarum* 571, *G. catenulatum* 162 or *S. candelabrum* 249 between 1.2×10^{4} and 6×10^{4} conidia of *B. allii* were produced cm^{-1} leaf surface. These antagonists significantly reduced conidial production by 84 to 97%. Only *G. roseum* 1813 did not reduce conidial production significantly. When the wetness period was interrupted 64 h after antagonists were applied, conidia production was reduced for by than 99%, so that no conidia could be detected, by all antagonists except *C. herbarum* 571, where a few conidia could be found, and *S. candelabrum* 249, where 3×10^{4} conidia cm^{-2} leaf surface were counted compared to 7×10^{5} conidia cm^{-2} leaf surface in the control treatment.

A close correlation was found between the log-transformed number of conidia of *B. allii* produced on dead onion leaves (5200 conidia cm^{-2} leaf surface as the average detection limit where considered when no conidia were found) and SI, calculated from the estimated leaf area covered with conidiophores of *B. allii* (Fig. 1).

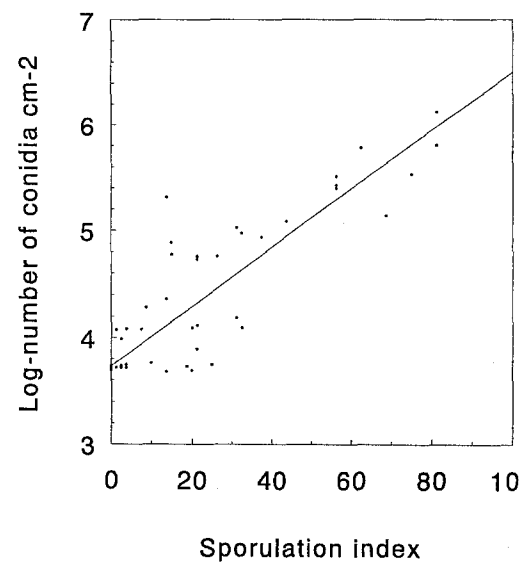

Fig. 1. Relationship between sporulation index (SI) estimated for leaf area of dead onion leaf segments covered with conidiophores of *B. allii* (y) and number of conidia of *B. allii* produced on dead onion leaf segments (x). Number of conidia per square centimetre was log-transformed and a density of 5200 conidia cm^{-2} was considered as the detection limit when no conidia were recovered. ($y = 3.730 + 0.028\ x$; $r = 0.915$; $p < 0.001$).

Bioassays with B. cinerea *with interrupted leaf wetness periods*

When conidial suspensions of *B. cinerea* (1×10^5 conidia ml^{-1}) were sprayed to the leaf segments 24 h before the antagonists *A. alternata* 317 and 319, *A. pullulans* 490, *C. globosum* 256, *G. catenulatum* 162, *G. roseum* 1813, and *U. atrum* 385 were applied as in bioassays with *B. allii*, SI values were higher than 90 on leaves not treated with antagonists. Antagonists did not reduce SI values significantly (data not shown). When *B. cinerea* was applied eight hours before the antagonist, antagonists were highly efficient if *B. cinerea* had been sprayed with 1×10^3conidia ml^{-1} (Table 4). The SI value was 63 for leaves not treated with antagonists, but was one or lower for leaves treated with antagonists. When *B. cinerea* was applied at 1×10^4 conidia ml^{-1}, the SI value was 64 for leaves not treated with antagonists. The antagonists reduced SI values to five or lower. At the highest concentration of *B. cinerea*, 1×10^5 conidia ml^{-1}, *C. globosum* 256 suppressed sporulation of *B. cinerea* completely; *A. alternata* 319 and *U. atrum* 385 reduced sporulation from a SI of 85 in the water treatment to 12 and 15, respectively. Treatments with *A. alternata* 317, *G. catenulatum* 162 and *G. roseum* 1813 were only moderately efficient, resulting in SI's between 32 and 53, and *A. pullulans* 490 showed no antagonistic activity. When wetness was interrupted after 16 h, both isolates of *Gliocladium* spp. showed only moderate or no antagonism at all concentration levels of *B. cinerea* (Table 4). *C. globosum* 256 was also highly effective after a dry period when *B. cinerea* was applied with 1×10^3 conidia ml^{-1}, but did not reduce sporulation of *B. cinerea* when *B. cinerea* was applied in the highest concentration with 1×10^5 conidia ml^{-1}. *A. alternata* 317 and 319 and *U. atrum* 385 showed no sensitivity to an interruption of leaf wetness and suppressed sporulation of *B. cinerea* as efficiently as under continuously wet conditions.

Water availability

From the relationship between water content and water potential in dead onion leaves (Fig. 2) and the gravimetrically measured water content of dead onion leaves during the drying process, it can be concluded that under our experimental conditions the water potential of dead onion leaf segments was lower than −5 MPa after approximately five hours and approximately −12 MPa after seven hours of drying (Fig. 3).

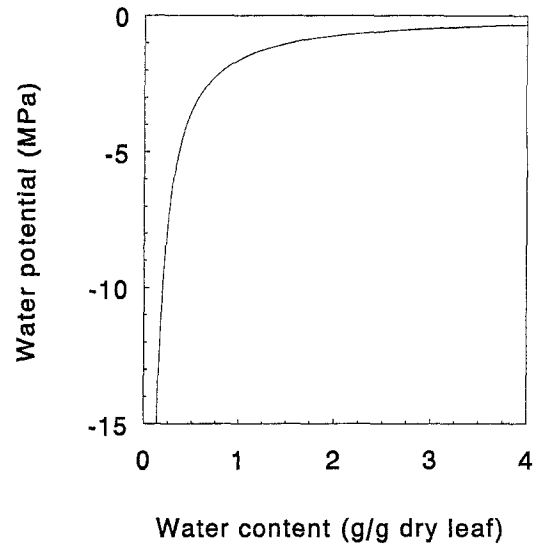

Fig. 2. Relationship between water content (y) and water potential (x) in dead onion leaves. ($y = -1.610 \times ^{-1.121}$; $r = 0.952$, $p < 0.001$).

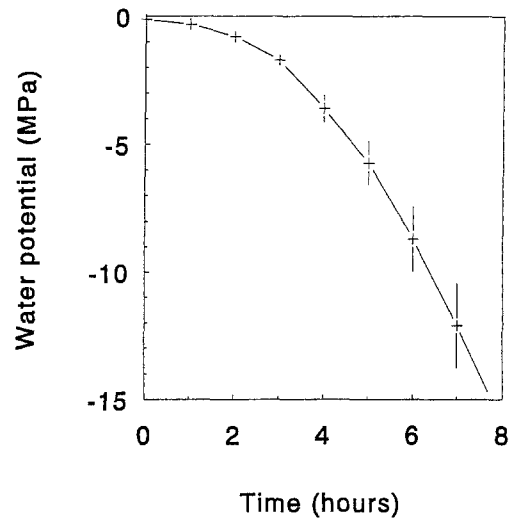

Fig. 3. Water potential of dead onion leaf segments during drying process in bioassays with an interrupted leaf wetness period. Water content of dead onion leaves was measured gravimetrically and water potential was calculated using formula given in Fig. 2. Bars indicate standard error of the mean.

Discussion

Necrotrophic pathogens such as *Botrytis* spp. sporulate exclusively on necrotic tissue caused by infection, senescence or other factors. *Botrytis* spp. typically colonize and subsequently sporulate on necrotic tissue of spreading lesions on leaves e.g. *B. elliptica* in lilies,

on remains of flowers e.g. *B. cinerea* in grapevine or on dead leaves [Braun and Sutton, 1987; Dubos *et al.*, 1982]. To suppress sporulation of necrotrophic pathogens, antagonists should be able to rapidly colonize such substrates in the presence of the pathogen and to successfully compete with the pathogen.

The bioassay method, based on interactions between the *Botrytis* spp. and antagonistic fungi on dead leaf tissue, effectively selected such antagonists. Onion leaves that senesced and dried naturally in the field were not suitable for standardized experiments because they frequently were already colonized by naturally-occurring saprophytes. Symptomless green leaves of field-grown onions, that were killed by drying and subsequently sterilized gave reproducible results in the bioassays. Senescing tissue of leaves attached to the plant contains less nutrients compared to a healthy leaf because during senescence nutrients are translocated to younger tissue [Baddeley, 1971] or because soluble nutrients are leached during rainfall. Therefore, leaf segments used in bioassays were thoroughly washed to remove soluble nutrients to create a substrate comparable to naturally senesced tissue. Under field conditions, necrotic leaf tissue may be already partly be colonized by *Botrytis* spp. before antagonists arrive. Therefore, leaves used in the bioassays were inoculated with conidia of *Botrytis* spp. eight to 24 h before antagonists were applied to select for antagonists with strong competitive abilities.

The method used to estimate sporulation intensity of *Botrytis* spp. gave reproducible results and was less time consuming than counting conidia produced on the substrate per square centimetre. Because the estimated leaf area covered with conidiophores correlated closely with the log-transformed numbers of conidia produced on the substrate (Fig. 1), this method was reliable to detect distinct reductions of sporulation as were found in the bioassays. However, the method may not be effective when antagonists affect the sporulation index (SI) only slightly in the upper range of the scale. It is possible that moderately competitive antagonists suppress the number of conidia produced per conidiophore of *Botrytis* spp. whereas highly competitive antagonists suppress chiefly the number of conidiophores produced by the pathogen.

Under constantly humid experimental conditions most of the antagonists suppressed sporulation of *B. allii* efficiently in the initial screening experiments. Complete inhibition of sporulation of *B. allii* was common with a range of antagonists even when dead leaves had been incubated with the pathogen for 24 h before antagonists had been applied and the inoculum density of antagonists was only ten-fold of that of the pathogen. Only saprophytic fungi were included in the screening that had been isolated from necrotic leaf parts or that belong to a group of well-known antagonists, *Trichoderma* spp. and *Gliocladium* spp.. *Trichoderma* spp. and *Gliocladium* spp. have been shown to be antagonistic against a wide range of fungal pathogens [Papavizas, 1985] including *Botrytis* spp. [Dubos, 1992]. Saprophytes isolated from necrotic leaf tips of field grown plants had to compete with other naturally occurring saprophytes in the field during the fungal succession in the substrate. Thus, it could be assumed that such fungi show a high competitive saprophytic ability also in bioassays.

Antagonists with high competitive saprophytic ability in necrotic leaf tissue may be suppressive to a wide range of *Botrytis* spp. and necrotrophic pathogens. While *B. allii* was used in most of the experiments to select antagonists, antagonists showed the same differentiation of antagonism and sensitivity to interrupted wetness periods when tested with *B. cinerea* (Table 4). However, reduction of sporulation of *B. cinerea* was less compared to *B. allii* when applied 24 h in advance to antagonists.

The performance of antagonists under field conditions is determined mainly by their ecological competence. The environmental conditions of above ground necrotic leaves are harsh for the development of both pathogen and saprophytic antagonists. Water availability, extreme temperatures, UV-radiation and nutrient depletion are among the factors limiting fungal development [Burrage, 1971; Diem, 1971; Park, 1982; Rotem *et al.*, 1985]. Frequent and rapid fluctuations of water potential may be highly restrictive for fungal development in the field, where periods of leaf wetness or high humidity are usually interrupted daily by dry periods. In general, fungal hyphae, especially fungal germ tubes, are sensitive to dry periods, but fungi can essentially differ in their potential to regrow and in the lag time needed for regrowth [Park, 1982]. Antagonists aimed at suppression of sporulation of *Botrytis* spp. and other necrotrophic leaf pathogens on necrotic leaf tissue may only be reliable under field conditions if they are able to survive during dry periods and to start to regrowth rapidly with only short lag times after conditions become favourable for fungal growth again. Bioassays with interrupted leaf wetness periods were suitable to differentiate antagonists according to their sensitivity to dry conditions during the colonization process.

Two main factors determine the efficacy of antagonists after an interruption of leaf wetness: (1) their mycelial growth rates and competitive ability at low water potentials during the drying process and (2) their survival during, and rapid regrowth after, dry periods when water potentials of the substrate are too low to allow fungal growth. Under our experimental conditions, the water potential of the dead leaves decreased continuously after wet leaves had been placed on dry filter paper in open petri dishes placed in a laminar flow cabinet. The drying process lasted approximately 7 hours until the water potential was lower than −12 MPa, at which fungal growth is slow or stops [Alderman and Lacy, 1984; Magan and Lacey, 1984; Magan and Lynch, 1986; Hocking *et al.*, 1994]. As a consequence, antagonists sensitive to low water potentials may colonize the substrate to a lower extent and may not be as competitive as antagonists growing also at lower water potentials. Under the experimental conditions, antagonists and *Botrytis* spp. also had to survive several hours in the substrate at water potentials too low for fungal growth, so that both aspects of drought tolerance, growth at low water potential and survival during dry periods, were tested in combination in these bioassays.

Antagonists originating from necrotic leaf tissue such as *A. alternata* and *U. atrum* were highly antagonistic even after leaf wetness periods had been interrupted repeatedly on three consecutive days. On the other hand, soil-borne fungi such as *Gliocladium* spp. and *T. harzianum* showed sensitivity to interrupted leaf wetness periods especially during the early stage of colonization at the first day after application. Germinating spores and germ tubes of these fungi may be more sensitive to dry periods than well developed mycelium formed later during the colonization process.

Under Dutch climatic conditions, leaf wetness periods in the field can be shorter and the drying process can be more rapid than was realized in our tests [Köhl *et al.*, unpublished]. In the field, necrotic leaf tissue may dry in less than 1 h and dry periods can be much longer than the few hours as in our bioassays. Furthermore, leaf wetness periods shorter than 1–2 h frequently occur in the field e.g. on rainy, windy days, during which energy consuming regrowth of hyphal tips may be initiated without resulting in an uptake of new nutrients. The duration of wet periods may be an important factor for a further differentiation between antagonists with different ecological competence. Zhang and Pfender [1993] tested the effect of different wetting periods on the interaction between *Pyrenophora tritici-repentis* and several antagonists in wheat straw. Ascocarp formation of the pathogen was suppressed by antagonists when wetness periods lasted for 24 h or longer but not after wetness periods of 6 h or 12 h.

Antagonists could be selected in bioassays that substantially suppressed sporulation of *Botrytis* spp. on dead leaves of onion. By alternating wet and dry periods in the substrate, conditions in the field were simulated that may restrict development of fungi not ecologically adapted to that niche. Antagonists effective also after repeated wet-dry-cycles could be found. The effect of such isolates under field conditions on the sporulation of *Botrytis* spp. and on the progression of epidemics caused by the pathogen are tested in ongoing studies.

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