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Suppression of sporulation of *Botrytis* spp. as a valid biocontrol strategy

Jürgen Köhl, Wilma M. L. Molhoek, Carin H. van der Plas and Nyckle J. Fokkema
DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands

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Abstract

In this study, the hypothesis was tested that removal of substrate for sporulation of *Botrytis* spp. may lead to a retardation of an epidemic if the majority of the inoculum is produced inside the treated crop. Suppression of sporulation of *Botrytis* spp. could be an attractive option for biological control of *Botrytis* leaf spot in onions. In a field experiment, necrotic leaf tissue was removed to simulate the effect of a biocontrol agent. By this means, the amount of substrate on which *Botrytis* spp. sporulates was reduced. In the experiment, the spore load above the onion plots was significantly reduced and the epidemic of onion leaf spot was retarded. At the end of the growing season, the number of leaf lesions in the green leaf area was lower in plots with substrate removal than in control plots (0.6 and 1.1 cm^{-2}, respectively). The results demonstrated that an epidemic of onion leaf spot largely depends on the rate of inoculum production inside a crop. Thus, suppression of sporulation on necrotic leaf tissue is a valid control strategy that could be applied by using sporulation suppressing antagonists.

Introduction

Botrytis spp. infect above ground tissues and harvested products of a large number of protected and field crops and can cause serious yield losses [Maude, 1980]. To date, control of many *Botrytis* diseases depends chiefly on fungicides and it is common that fungicides are applied weekly in crops such as onions or flowerbulbs. Frequent use of fungicides, especially dicarboximides and benzimidazoles, results in populations of *Botrytis* spp. developing fungicide resistance [Gullino, 1992]. The capacity of *Botrytis* spp. to develop fungicide resistance underscores the need for fungicide management that retards resistance build up. Disease management that incorporates biological control would be expected to limit the selection pressure on *Botrytis* populations and the development of fungicide resistance. Furthermore restrictive regulations concerning the reduction in the use of chemical pesticides urge alternative control methods. Biological control is potentially an environmentally friendly alternative or supplement to chemical control.

To date, biological control of *Botrytis* spp., as recently reviewed by Dubos [1992], has been aimed mostly at the protection of green leaves against infection by the pathogen. In the phyllosphere however, there is a short interaction time in the order of a few hours between germinating conidia of *Botrytis* spp. and antagonists and the pathogen may escape from antagonism by penetration of the healthy plant tissue. This probably limits the feasibility of this control strategy, which mainly imitates the protection of plants with fungicides [Fokkema, 1993; Köhl and Fokkema, 1994]. The choice of a biological control strategy needs to be based on the ecology of both the pathogen

and the antagonist. Necrotrophic pathogens such as *Botrytis* spp. infect healthy plant tissue but mycelial growth and production of conidia occurs in necrotic tissue after necrosis has been induced either by *Botrytis* or by other factors. Epidemics produced by *Botrytis* spp. in the field usually are polycyclic, and involve cycles of infection and sporulation. A control strategy based on competition of saprophytic antagonists with saprophytically growing mycelium of *Botrytis* spp. in necrotic plant tissue, aims at reducing growth and sporulation of the pathogen. In this way, the reduction of sporulation of *Botrytis* spp. may result in a retardation of the polycyclic epidemic of the disease. A potential advantage of this approach for biological control of *Botrytis* spp. is the long interaction time between pathogen and antagonist. Furthermore, necrotic plant tissue is a natural substrate for saprophytic fungi and can enable antagonists to develop high population densities after substrate colonization and so limit or even eliminate the inoculum sources of *Botrytis*. Several studies have demonstrated the validity of microbial suppression of colonization and sporulation as a biocontrol strategy [Köhl and Fokkema, 1994; Sutton and Peng, 1993].

Exploiting sporulation suppression of necrotrophic pathogens as a control strategy demands more knowledge of the rate of disease development in the crop compared to a strategy aiming at prevention of infection. In the latter case, a possible control effect can also be demonstrated directly on single plants or even a single leaf. Suppression of sporulation as a strategy for biological control is only feasible (1) when the progression of an epidemic depends on the amount of inoculum in the crop, and (2) when the inoculum is produced mainly inside the antagonist-treated crop and air-borne inoculum from outside the crop does not contribute substantially to the disease progress. The objective of this study was to test, under field conditions, the effectiveness of sporulation suppression as a strategy for biological control of *Botrytis* spp. The polycyclic epidemic of onion leaf spot, caused by *B. cinerea* Pers. ex Pers. and *B. squamosa* Walker, was chosen as a model. Suppression of sporulation by antagonists was simulated by the artificial removal of necrotic onion leaf tissue on which *Botrytis* spp. can sporulate. The effect of removing necrotic tissue on the spore load of *Botrytis* spp. in the crop and on the development of leaf lesions was followed. In an additional treatment, *Gliocladium roseum* was applied to test the capacity of this antagonist to suppress sporulation of *Botrytis* spp. under field conditions.

Materials and methods

Field plots. Onion cv. Hyton F1 was sown in rows 0.3 m apart on 27 March 1991 in 18 plots of 9 × 12 m, arranged in six blocks of three plots each. Three treatments were allocated at random within each block, so as to give a randomized block design in six replications. Plant density, determined on 13 April, was 93 plants m^{-2}. In order to minimize interplot interference, the plots were separated by 12 m wide strips of sugar beet. Sugar beet was chosen as buffer crop because it does not produce pollen which could stimulate *Botrytis* infection and is not sufficiently tall to cast shadow on the plots which would alter the microclimate in the onions.

Inoculum source. Sclerotia of *B. squamosa*, the primary inoculum in epidemics of onion leaf blight [Ellerbrock and Lorbeer, 1977], were produced on sterile onion leaves placed on water agar. Sclerotia were separated from the leaf tissue using forceps and 2.4 sclerotia m^{-2} were spread on 23 April by hand over the plots in order to obtain a homogeneous distribution of primary inoculum of *B. squamosa* at the beginning of the season.

Treatments. The effect of the suppression of sporulation of *Botrytis* spp. by antagonists was simulated by artificial removal of necrotic leaf tissue. Therefore, all onion leaves with important necrotic area (necrotic for more than 50% of their length) were cut off using scissors and removed from the plots at weekly intervals from 5 June till 9 August. Removing necrotic leaf tips also from young leaves (necrotic for less than 50% of their length) might have led to infections and physiological stress of onion plants. Since the amount of necrotic tissue increased drastically at the end of July, the removal of necrotic leaf tissue could only be continued in two arbitrarily chosen blocks.

A second treatment consisted of the application of the fungal antagonist *Gliocladium roseum* Bain IPO-1813, that had been isolated from the surface of a potato tuber. In bioassays based on interactions on dead onion leaf segments under humid conditions, *G. roseum* 1813 showed strong antagonism against *B. aclada* Fres. (syn. *B. allii* Munn) and suppressed sporulation of this pathogen completely [Fokkema *et al.*, 1992]. The antagonist was sprayed as conidial suspension at weekly intervals from 5 June till 14 August at a volume of 60 ml m^{-2}. Conidia were produced on autoclaved moist wheat grains in 250 ml conical flasks (30 g dry weight of grains per flask) during 14 days at 18 °C. Suspensions were prepared by adding tap water with 0.01% Tween 80 to the cultures. The suspension was shaken, filtered through a double layer of cheesecloth and the conidial concentration was adjusted to approximately 1×10^6 conidia ml^{-1}. No treatments were carried out in the control plots.

Observations

Necrotic leaf tissue. In all treatments, ten randomly chosen plants (each with 4 to 6 leaves) per plot were sampled at weekly intervals from 11 June till 20 August. The length of green and necrotic leaf parts was measured to estimate the efficacy of the removal of necrotic tissue and to determine the effect of the treatment with *G. roseum* on leaf dieback caused by *B. squamosa.* Since necrosis of leaf tissue is a continuous process during the interval between sampling dates, and since removal of necrotic tissue with more than 50% necrotic leaf length took place at different dates after sampling, the percentage necrotic area which had been removed could only roughly be estimated at about 60% (Fig. 1).

Spore load . Spore samplers (Rotorods, Sampling Technologies, Los Altos Hills, CA, USA) were used to trap airborne spores [Edmonds, 1972; Aylor, 1993]. These were positioned at a height of 0.3 m in the centre of each plot of the two blocks where necrotic leaf tissue was removed during the whole duration of the experiment. Thus, spores were trapped simultaneously in all treatments in two replicates. Additional sampling was carried out to compare density of airborne spores within and outside the plots. For this, Rotorods were placed 0.3 m above the centre of a control plot, within the buffer crop between a control plot and a plot treated with *G. roseum* and within

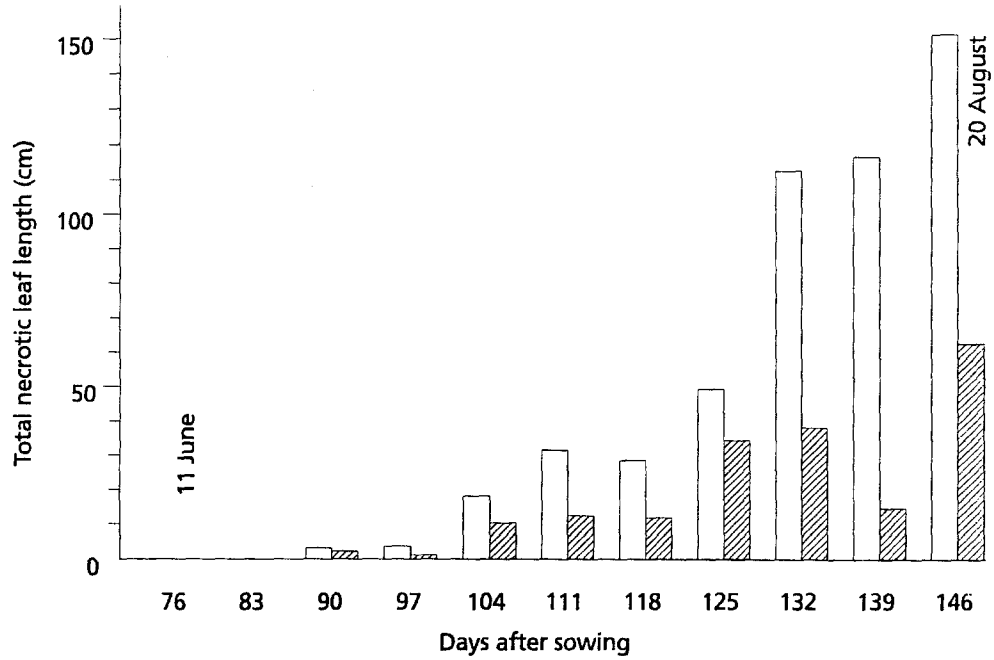

Fig. 1. Total necrotic leaf length per onion plant after removal of leaves with more than 50% necrotic leaf length (▨) in comparison to the control treatment (□). Data of 2–6 replications with 10 plants each per sampling date.

the buffer crop at a distance of 18 m from onion plots.

Between 1 and 21 August, spores were trapped on five days. Three, three, four, two, and three sampling runs, each of 15 min., were carried out on 1, 10, 12, 16, and 21 August, respectively. All runs were done between 11:00 hours and 15:00 hours. Peaks of spore release could be expected at this time of the day coinciding with a decrease in humidity within the crop [Sutton *et al.*, 1978]. Conidia were counted on a length of 22 mm of each of the two rods of a Rotorod per sampling run. The numbers of *Botrytis* conidia shorter than 15 µm and those longer than 15 µm were recorded separately in order to distinguish between conidia of *B. cinerea* and *B. squamosa*. According to Ellis [1971] and our own measurements of conidial sizes of several isolates of *B. cinerea* and *B. squamosa* grown on sterile onion leaves, conidia of *B. cinerea* usually are 8 to 14 µm long and those of *B. squamosa* usually are 15 to 21 µm long. The concentration of conidia m^{-3} air was calculated from the conidial counts, the rotation speed and the sampling duration, according to a formula provided by the manufacturer. Under the prevailing sampling conditions, one hundred conidia counted per sampling run on the two rods together represent 147 conidia m^{-3} air.

Disease development. Lesions were counted on the green parts of all leaves of the plants except those of which the necrotic leaf part exceeded 50% of the leaf length and which had been removed as part of the experiment. The surface of the green leaves was measured with a Delta-T Area Measurement System (Delta-T Devices LTD., Burwell, Cambridge, UK) and the number of lesions cm^{-2} was calculated.

Microclimate. Air temperature, relative humidity and leaf wetness duration were measured in the onion crop at 0.35 m height continuously at 60 min intervals using a Delta-T logger (Delta-T Devices LTD., Burwell, Cambridge, UK) connected with a Valvo-sensor (Philips, Eindhoven, The Netherlands) and a leaf wetness sensor (LW 100, Bottemanne, Amsterdam, The Netherlands).

Data analysis. The statistical package Genstat 5 was used for data analysis [Genstat 5 Committee, 1990]. The number of conidia trapped during all sampling runs were pooled per day and analyzed for conidia of *B. cinerea* and *B. squamosa* separately. After analysis of variance of log-transformed data, differences between treatments were established with LSD-tests.

Progressive increase in number of lesions cm^{-2} leaf was modelled for each plot separately. The exponential growth model $y = \exp(a + bt)$, with y = density of lesions, t = time, a = log (initial density) and b = relative growth rate (RGR), proved to be adequate. Values of a and b for each plot were subjected to analysis of variance.

Results

Incidence of Botrytis *spp.*

After introducing sclerotia of *B. squamosa* into the field on 23 April, weather was dry for two weeks. No sporulation of *B. squamosa* was found on the introduced sclerotia and many of the sclerotia were attacked by collembola (e.g. *Sminthurus* spp.). After 21 days, no sclerotia could be recovered. Thus, it can be assumed that most infections of *Botrytis* during the field experiment were caused by naturally occurring inoculum. The appearance of lesions caused by *B. cinerea* or *B. squamosa* is rather similar and does not allow a conclusion on the pathogen involved. A majority of conidia of *Botrytis* found on dead leaf tips during the growing season or collected with Rotorods at all trapping periods was smaller than 15 µm, most probably belonging to *B. cinerea* (Fig. 2). Overall, only 12.7% of trapped conidia were larger than 15 µm and conformed with the size of those of *B. squamosa*. *B. cinerea* was isolated from a large number of lesions of onion leaves, sampled regularly during the field experiment, whereas *B. squamosa* was never isolated.

The sugar beet buffer crop was inspected at regular intervals visually for sporulation of *Botrytis* spp. Leaves of sugar beets remained healthy during our experiment, only a small amount of necrotic leaf tissue was found in the sugar beet crop. Sporulation of *Botrytis* spp. was never observed.

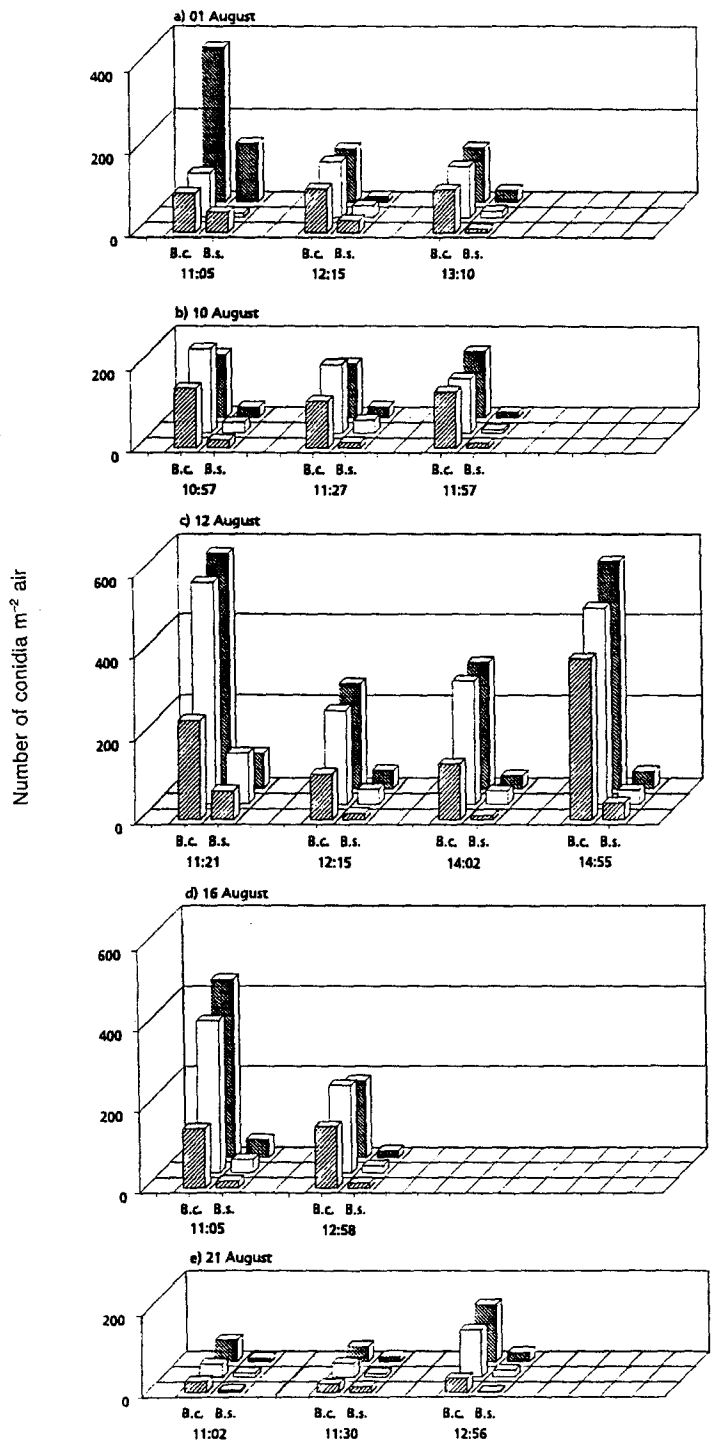

Fig. 2. Concentration of airborne conidia of *Botrytis cinerea* (B.c.) and *B. squamosa* (B.s.) above onion plots from which necrotic leaf tissue was removed at weekly intervals (▨), above plots that were sprayed with conidial suspensions of *Gliocladium roseum* (□), and above control plots (▩). Each bar represents a mean value for two replications per treatment. a–e. Five different dates; local time indicated below the x-axis. Concentrations of airborne spores of *B. cinerea* in the control plots and the plots where necrotic tissue had been removed differed significantly ($P < 0.05$) on 12 and on 16 August.

Spore load

From the beginning of August onwards, sporulation of *Botrytis* spp. was abundant on necrotic leaf tips after periods with persistant dew. In experimental plots where approximately 60% of the necrotic tissue had been removed, the spore load of conidia of *Botrytis* spp. was generally lower compared to the control (Fig. 2). Concentrations of airborne spores of *B. cinerea* in the control plots and the plots where necrotic tissue had been removed differed significantly ($P < 0.05$) on 12 and on 16 August. On these dates the average concentration of *B. cinerea* conidia above plots from which necrotic tissue was removed was 50% less than that above the control plots. Treatments with *G. roseum* did not significantly affect the spore load of *B. cinerea*.

The spore load above the buffer crop, between the plots, at a distance of 6 m from the nearest onions, was on average 55% of the spore load above the onion crop. At a distance of 18 m from onions, the spore load was 40% of the spore load above the onion crop.

Leaf lesions

A few *Botrytis* lesions were observed in the first two weeks of June but no differences were found among the treatments. Until the middle of July, the weather was dry and warm without dew formation during nights and epidemics of *Botrytis* did not progress. Thereafter, rainy weather and dew formation during nights favoured the development of onion leaf spot and the number of lesions increased exponentially (Fig. 3). The initial densities did not differ significantly among treatments, but RGR for the plots where necrotic leaf tissue had been removed was significantly smaller ($P < 0.05$) than for the control and antagonist treatment. Since block differences were not significant, the block effect was pooled with the residual effects. At the last sampling date on 20 August, the number of lesions cm^{-2} of green leaves was 1.1

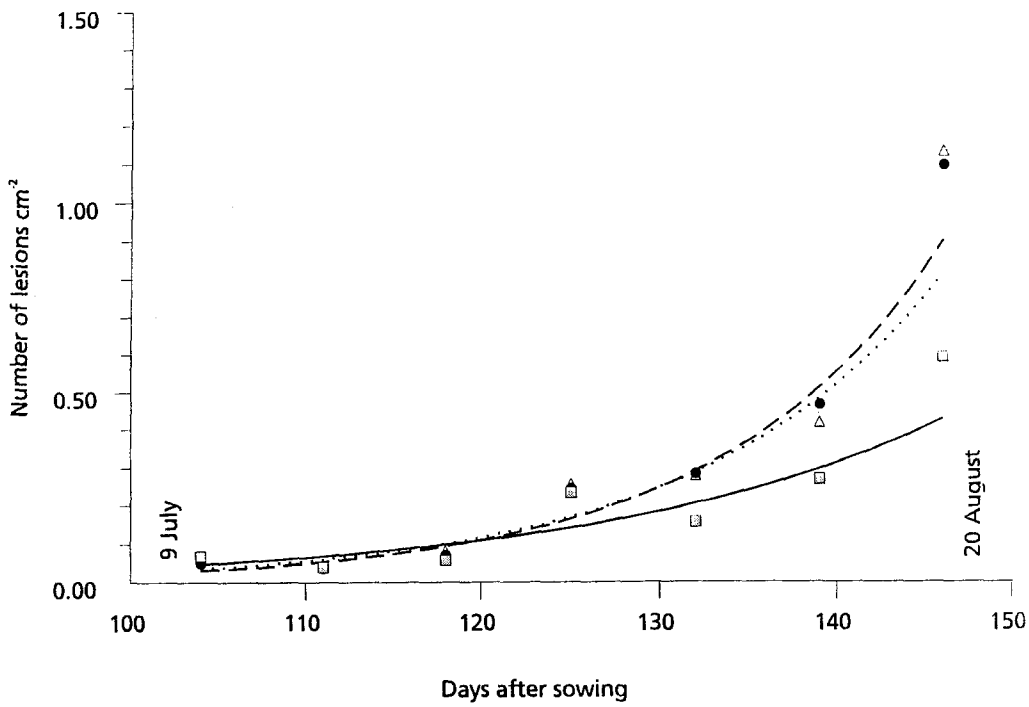

Fig. 3. Number of leaf lesions caused by *Botrytis* on green onion leaves in plots from which necrotic leaf tissue was removed at weekly intervals (▨ ——), in plots sprayed with *Gliocladium roseum* (● · · · ·), and in control plots (△ – –). Means of 2–6 replications with 10 plants each per sampling date; curves were fitted with exponential growth model y = exp (a + bt). Relative growth rate (parameter b) for plots from which dead leaf tissue was removed was significantly different ($P < 0.05$) from plots treated with *G. roseum* and control plots.

in the control, 1.1 in the antagonist treatment and 0.6 in the plots where necrotic leaf tissue had been removed. The treatment with *G. roseum* had no effect on the amount of necrotic leaf tissue.

Discussion

The artificial removal of necrotic leaves reduced the spore load of *B. cinerea* above the experimental plots and retarded the epidemic of onion leaf spot, compared to the control treatment. The relationship between the amount of necrotic tissue as substrate for *Botrytis* to sporulate on, the spore load above a plot and number of leaf lesions, indicated that the progression of the onion leaf spot epidemic largely depended on the rate of inoculum production inside the crop. From these relationships it can be concluded that application of antagonists to suppress sporulation of *Botrytis* spp. on necrotic leaf tissue could be an effective strategy for biocontrol.

The counts of spores recovered in traps positioned between the experimental plots and at a distance of 18 m from onion plots indicated that there was a gradient in the concentration of airborne spores for several meters away from diseased onions. Consequently, even though the plots were spatially separated by a 12-m buffer strip of sugar beet, interplot interference may have occurred during the experiments. A treatment at field level without interplot interference from nearby untreated control plots could have resulted in an even greater disease suppression in response to reduced spore production in the plots.

B. cinerea, an ubiquitous, non-host-specific pathogen, was the chief pathogen in the field experiment. Conidia of *B. cinerea* are common atmospheric microbes [Gregory and Hirst, 1957]. Main sources of conidia of *B. cinerea* are diseased plants of field crops or the natural vegetation, plant debris and sclerotia. A certain background level of airborne conidia of *B. cinerea* may always be present even when no disease is present in the crop. Inoculum produced outside a crop may initiate an epidemic in a field crop, but once an epidemic has started and sporulation occurs inside the field, airborne inoculum from outside the field becomes relatively less important as shown by our experiment.

For other crops such as strawberries and raspberries it has been demonstrated that necrotic plant tissue inside the crop serves as the main inoculum source during an epidemic of *B. cinerea* [Braun and Sutton, 1987, 1988; Jarvis 1962a, 1962b, 1962c; Jordan and Pappas, 1977; Miller and Waggoner, 1957]. In kiwifruit vines, senescing male flowers and prunings on the ground were found to be the main sources of inoculum of *B. cinerea* [Elmer *et al.*, 1993]. A significant correlation was found between the inoculum potential inside a plot and the external kiwifruit contaminations.

Control of polycyclic diseases can be aimed at reduction of either initial or subsequent inoculum. Disease control may only be achieved when the progression of an epidemic mainly depends on inoculum density and when main sources of inoculum can be reached by control agents. Reduction of inoculum of *Botrytis* spp., that is produced outside the crop is not feasible because inoculum sources are spread over large areas, but inoculum produced inside the crop, such as on crop debris or on sclerotia, could be accessible for biological control. Antagonists such as cold tolerant *Trichoderma* spp. may destroy overwintering sclerotia [Köhl and Schlösser, 1989]. A reduction of initial inoculum may delay disease development. Adee and Pfender [1989] demonstrated a positive relationship between the amount of local initial inoculum of *Pyrenophora tritici-repentis* and the disease progress curve of tan spot in wheat in field experiments with different levels of primary inoculum. However, a 60–80% reduction of ascospore formation as reached with applications of the antagonist *Limonomyces roseipellis* was not sufficient to control tan spot because of secondary conidiation [Pfender *et al.*, 1993]. For three other polycyclic pathosystems including *B. cinerea* on *Begonia semperflorens*, Plaut and Berger [1981] found that initially low inoculum densities were compensated for by higher rates of disease increase compared to initially high inoculum densities. Thus, reduction of initial inoculum alone by biological control or other means may not always result in efficient disease control.

Control strategies aimed at the reduction of the initial inoculum and subsequent conidial inoculum of a polycyclic pathogen may be more successful

because they interfere with the multiplication of the pathogen during the whole epidemic. Control of *B. cinerea* by suppressing sporulation was suggested by Jarvis [1962a, 1962c] by using a chemical antisporulant or by means of plantation hygiene, which may limit potential sporulation sites for *B. cinerea*. Jordan and Pappas [1977] suppressed sporulation of *B. cinerea* in the field by spraying fungicides on strawberry debris before flowering. Consequently, the number of conidia trapped with Rotorods inside treated plots during flowering was drastically reduced and strawberry fruit rot was reduced with 66%. Also Braun and Sutton [1986] controlled strawberry fruit rot by pre-blossom fungicide applications on dead host leaves. The control of grey mould in grapevine [Dubos, 1987; Gullino and Garibaldi, 1988] and cucumber [Elad *et al.*, 1993] by *Trichoderma* spp. is often assumed to be based on competition for infection-stimulating nutrients, such as flower petals and pollen, but suppression of sporulation on these substrates could be involved as well, since *Trichoderma* spp. may live saprophytically in necrotic tissue.

The isolate of *G. roseum* 1813 used in our field experiment suppressed sporulation of *B. aclada* completely in bioassays on dead onion leaves at conditions of saturated humidity in moist chambers [Fokkema *et al.*, 1992]. However, under field conditions, the isolate had no effect on the leaf spot epidemic in onions. The antagonist could not be reisolated from necrotic leaf tissue. *G. roseum*, primarily a soil inhabitant, may not be ecologically adapted to micro-climatic conditions in necrotic leaf tips of onions. The discrepancy of our results with the success of *G. roseum* in suppressing sporulation of *B. cinerea* in strawberry leaves [Sutton and Peng, 1993] may be explained by the endophytic colonization of *G. roseum* in green strawberry leaves by which it escapes from the harsh environmental conditions in the phyllosphere [Sutton, pers. comm.]. After leaf senescence, the antagonist may immediately compete with saprophytically growing mycelium of *B. cinerea*.

Recent bioassays at our laboratory are focused on sporulation suppression under alternating humid and dry conditions. In these tests several isolates of *Alternaria* spp., *Chaetomium globosum* and *Ulocladium* spp., all obtained from plant debris, performed much better than *G. roseum* [Köhl *et al.*, 1993]. Yeasts were also found to suppress both conidial germination and sporulation of *B. cinerea* [Elad *et al.*, 1994]. These results encourage further exploration of biocontrol based on sporulation suppression.

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