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# The buffering capacity of the natural mycoflora of rye leaves to infection by Cochliobolus sativus, and its susceptibility to benomyl

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**Abstract**

Different densities of saprophytic colonization of rye leaves were obtained in field experiments by spraying with benomyl or water. In 1972 and in 1973, inoculation with *Cochliobolus sativus*, just after flowering, resulted in 60% less necrosis on water-sprayed leaves than on benomyl-sprayed leaves. At that time, the natural mycoflora of the water-sprayed leaves amounted to 10 000 and 3000 propagules per $cm^2$ leaf surface in 1972 and 1973, respectively. The benomyl treatment reduced the colonization to 1200 and to 400 propagules per $cm^2$ in 1972 and 1973, respectively, which implied an apparent reduction of the antagonistic capacity of the mycoflora. In 1974, the saprophytic colonization of the water-sprayed leaves reached only 500 propagules per $cm^2$ just after flowering, a population density not high enough to be antagonistic. Benomyl had a differential effect on the phyllosphere fungi: *Cryptococcus* spp. were not affected, *Sporobolomyces roseus* and *Cladosporium* spp. were reduced to less than 6% of the control populations, and *Aureobasidium pullulans* was eliminated. When later in the seasons of 1972 and 1974 the 'white yeasts' on the benomyl-sprayed leaves reached population densities of 6500 propagules per $cm^2$ and more, inoculation of these leaves resulted in a necrosis similar to that of the water-sprayed leaves with higher population densities. Above a population density of 6500 propagules per $cm^2$ no correlation existed between the density of the antagonists and their action on *C. sativus*.

## Introduction

Antagonistic interactions between common phyllosphere fungi (yeasts included) and perthotrophic pathogens have been demonstrated in glasshouse experiments (Bhatt and Vaughan, 1962; Akai and Kuramoto, 1968; Van den Heuvel, 1969 and 1970; McBride, 1971; Warren, 1972b; Fokkema, 1973; Fokkema and Lorbeer, 1974). Information concerning biological control in the field, however, is very limited. Only Bhatt and Vaughan (1962) obtained a decrease in *Botrytis* infection of strawberries in the field by spraying with a conidium suspension of *Cladosporium herbarum*.

The greatest difficulty in biological control in the field could well be the maintenance of a sufficiently high population of antagonists. In nature, dense populations of saprophytes can develop before the onset of senescence on leaves of anemophilous crops. The saprophytic mycoflora on these leaves is stimulated by nutrients released from pollen deposited on the leaf after flowering (Fokkema, 1971; Warren, 1972a). In a rye field, pollen also stimulated leaf infection by *Cochliobolus sativus*, but only for a very short period after flowering. A simultaneously increasing saprophytic mycoflora probably interfered with the pathogen. Indications of such an antagonistic interaction were obtained in glasshouse experiments. The 'pollen effect' on *C. sativus* could be re-

duced by more than 50% when the inoculum was supplemented with isolates of the dominant phyllosphere fungi viz. *Cladosporium herbarum*, *Aureobasidium pullulans*, *Sporobolomyces roseus* and *Cryptococcus* spp. (Fokkema, 1973).

The present investigation was undertaken to demonstrate the existence of a buffering capacity of the natural mycoflora of rye leaves to infection by *C. sativus* and to study its susceptibility to fungicide interference. To achieve different levels of saprophytic colonization, rye leaves were sprayed with benomyl or water. If the saprophytes are of importance as antagonists, a prolonged stimulatory effect of pollen on *C. sativus*, which is relatively insensitive to benomyl (Bollen and Fuchs, 1970; Greenaway, 1973), could be expected. Experiments were performed in 1972 and repeated in 1973 and 1974.

## Materials and methods

*The host.* Spring rye, *Secale cereale* L. 'Petkuser' was grown on humous sandy soil. The experimental field measuring 15 × 15 m was divided in two halves for fungicidal and control treatments (Fig. 1). At 4 locations rye was grown in clusters of c. 25 plants allowing coverage with inoculation chambers. The clusters received the same fungicide treatments as the surrounding rye plants.

*The pathogen.* Conidia of the *Drechslera* state of *Cochliobolus sativus* (Ito & Kuribayashi) Drechsler ex Dastur, isolated from barley (Fokkema, 1971), were used as inoculum. After incubating for 12 days at 23 °C in darkness, on PDA (in 1972 and 1973) or oatmeal slants (in 1974), conidium suspensions were obtained by pouring 5 ml 0.1%

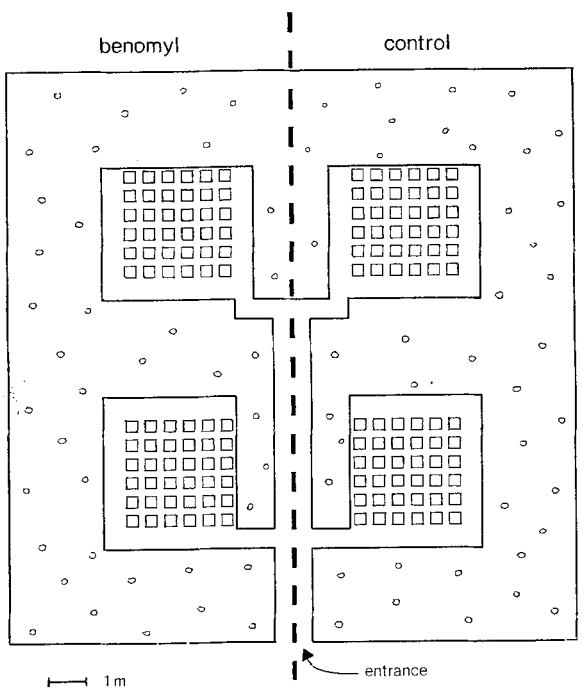
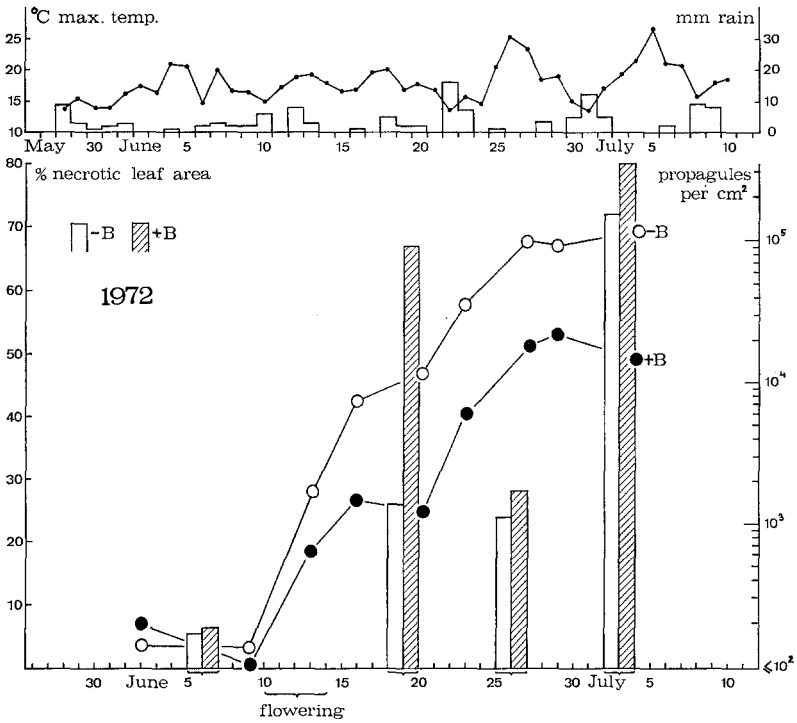
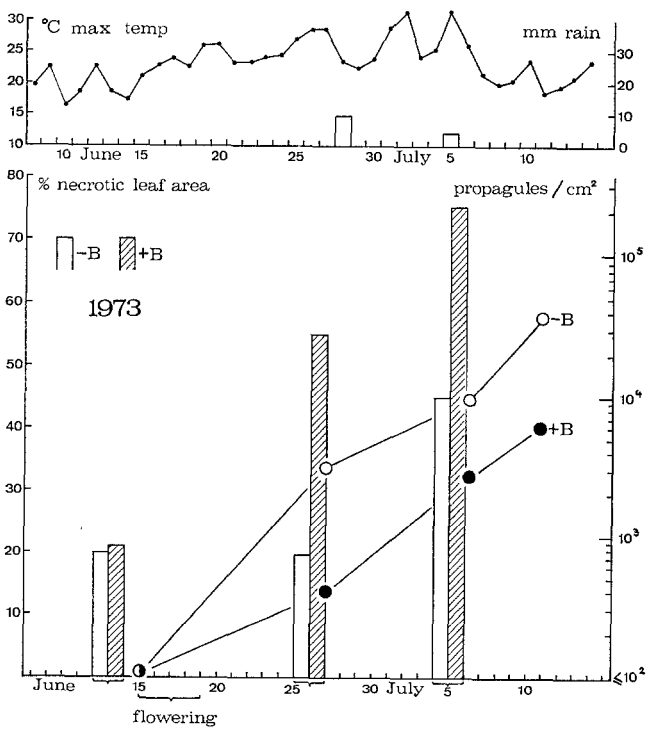



Fig. 1. Survey of the experimental field. At 4 locations rye was grown in clusters (□) of c. 25 plants, in the surrounding area rye was sown in rows (o).

*Fig. 1. Overzicht van het proefveld. Op 4 plaatsen groeide de rogge in groepjes (□) van circa 25 planten, in het omliggende gebied was de rogge in rijen gezaaid (o).*


°C max. temp.
mm rain
May 30 June 5 10 15 20 25 30 July 5 10
% necrotic leaf area
propagules per cm²
-B +B
1972
-B
+B
30 June 5 10 15 20 25 30 July 5 10
flowering


°C max temp
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% necrotic leaf area
propagules / cm²
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flowering

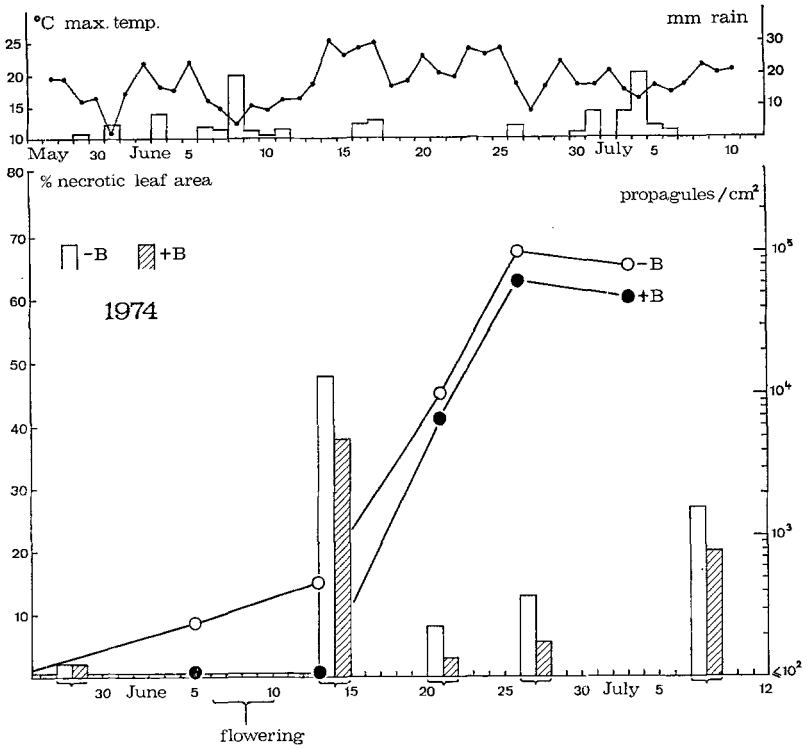


Fig. 2. Seasonal development of the total mycoflora of rye leaves sprayed with benomyl (+B) or water (−B) and its effect on the infection by *Cochliobolus sativus* in 1972, 1973 and 1974. The dots show the total number of saprophytic propagules per cm² (log scale) of benomyl- (●) or water-sprayed (○) leaves on successive dates. Columns, figured on the date of inoculation, show the percentage necrotic area of leaves treated with benomyl (hatched) or water (open) after inoculation at different dates.

Maximum daily air temperature and rainfall in the field are also recorded.

*Fig. 2. Ontwikkeling van de totale mycoflora op roggebladeren bespoten met benomyl (+B) of water (−B) en haar effect op de infectie door Cochliobolus sativus in 1972, 1973 en 1974. De punten geven weer het totale aantal saprofytische propagula per cm² (log. schaal) van met benomyl (●) of met water (○) bespoten bladeren op opeenvolgende data. De kolommen, getekend op de dag van inoculatie, hebben betrekking op het percentage necrotisch oppervlak vqn bladeren behandeld met benomyl (gearceerd) of water (open) na inoculatie op verschillende tijdstippen.*

*De dagelijkse maximum temperatuur van de lucht en de regenval in het veld zijn eveneens weergegeven.*

Tween 80 solution over the culture, scraping the cultures with an inoculation needle and shaking. Large fragments of mycelium were removed by filtration through glasswool. With the aid of a haemocytometer conidium concentrations were adjusted to 5–7 × $10^5$ conidia per ml.

*Inoculation technique and disease assessment.* Before flowering and at different

times after flowering the upper side of penultimate leaves of plants grown in clusters were sprayed with a conidium suspension of *C. sativus* until run off. On an average 14 leaves from 2–3 clusters of the benomyl-treated and of the control field were inoculated at each inoculation date (Fig. 2). After inoculation, transparant PVC inoculation chambers lined with moist filter paper were placed over the plants. At the end of each afternoon the filter paper and the inoculated leaves were wetted with a mist of water so as to ensure optimal conditions for infection. Above the chambers black plastic netting was spread to reduce solar irradiation. Temperature and relative humidity inside the chambers were registered utilizing a hygrothermograph. During bright weather the front door and the roof of the chambers were set ajar to avoid high internal temperatures. Under extreme weather conditions, temperatures and relative humidities inside the inoculation chambers varied from 7–35 °C and from 95–40 % r.h. During the night, relative humidities of more than 90 % were registered for at least 10 h.

The inoculated leaves were harvested 9–16 days after inoculation, depending on the progress of infection in a particular experiment. As parameter for the degree of infection the necrotic area of a leaf was determined with a planimeter and expressed as a percentage of the leaf surface. Differences in necrotic area between treatments were analyzed with Wilcoxon's two sample test.

*Treatment with benomyl.* Benlate (50 % benomyl) supplemented with 0.1 % Tween 80, was sprayed, 1.5 kg/ha, over one half of the experimental field. On the control half of the field, leaves were sprayed with water with 0.1 % Tween 80 added as a detergent. The sprayings were carried out on 21 April, 5 May, and 2 June 1972; on 18 May, 25 May and 7 June 1973; and on 13 May, 31 May and 19 June 1974.

*Analysis of the phyllosphere microflora.* The colonization of the leaves by saprophytic fungi was analyzed by culturing propagules (conidia, hyphal fragments and yeast cells) washed from the leaves (Fokkema, 1971). At each sampling date 8 leaves from benomyl-treated and from control plants were collected. They were first chopped and then vigurously shaken in erlenmeyer flasks, separately or together, with sterile water for 1 h. After appropriate dilution, aliquots of 0.1 ml were spread over the dry surface of cherry agar plates (in 1972 and 1973) or on PDA plates (in 1974), both supplemented with sodium benzyl-penicillin (50 iu/ml) and streptomycin sulfate (100 iu/ml). In 1974, nutrient agar plates with 50 ppm cycloheximide were also used for analysis of the bacterial leaf flora. After incubation at 23 °C for 3 days, the colonies were counted and classified as *Cladosporium* spp., *Aureobasidium pullulans*, 'pink yeasts' (mainly *Sporobolomyces* spp.), 'white yeasts' (mainly *Cryptococcus* spp.) and bacteria. The population density was expressed as the number of propagules per $cm^2$ of upper and lower leaf surface.

In 1972, a part of the shaking fluids was purified three times by centrifugation to recognize any disturbing effect of benomyl residues on the development of the microorganisms on the agar plates. With and without cleaning of the shaking fluids, similar numbers of colonies of all four fungal groups were obtained.

Rainfall was determined with a recording rain gauge. Temperature records were supplied by the Royal Dutch Meteorological Institute (KNMI, 1972, 1973, 1974), De Bilt, at a distance of c. 15 km from the experimental field.

## Results

*Relation between the total mycoflora and the degree of infection.* The development of the total mycoflora and the infection resulting from inoculations at different times are visualized in Fig. 2. In the three years inoculation before flowering caused less necrosis than inoculation after flowering (with one exception in 1973) because pollen deposited on the leaves stimulated the infection by *C. sativus*. The degree of infection of the benomyl-treated leaves was much higher than that of the control leaves, immediately after flowering in 1972 ($P \leqslant 0.01$) as well as in 1973 ($P \leqslant 0.05$). These differences of infection coincided with differences in the density of the saprophytes at that time. It seems that population densities of c. 10000 propagules $cm^2$ leaf surface in 1972 and of c. 3000 propagules/$cm^2$ in 1973 were high enough to reduce infection. In 1974, no significant difference in infection was noticed between leaves treated with benomyl or water. Probably a population density of 500 propagules/$cm^2$ on the control leaves was too low to exert an apparent antagonistic effect.

The second inoculation after flowering in 1972 on 26 June did not result in a difference in infection between the benomyl- and water-sprayed leaves. The infection of the benomyl-sprayed leaves was considerably less than after the foregoing inoculation ($P \leqslant 0.01$). The population densities of the saprophytic fungi on both benomyl- and water-treated leaves, by this time reached more than 10000 propagules/$cm^2$. At these high population densities no correlation could be found between the density of the saprophytes and the antagonistic effects. In 1973, the second inoculation after flowering on 5 July resulted again in different degrees of infection ($P \leqslant 0.05$) on benomyl- and water-sprayed leaves. The population density on the benomyl-sprayed leaves was only 2600 propagules/$cm^2$, whereas on the control leaves the mycoflora reached a density of 10000 propagules/$cm^2$. In 1974, on 21 June both benomyl- and water-treated leaves had population densities between 6500 and 10000 propagules/$cm^2$. Both groups of leaves had very low degrees of infection, which did not differ significantly.

In 1972 and 1974 further inoculations were carried out later in the season. The population densities of saprophytes were always above 10000 propagules/$cm^2$ and no differences in infection were noticed. In 1972, senescence of the leaves probably interfered with the infection process leading to very high ratings of necrosis.

The colonization of the rye leaves by bacteria was given attention in 1974. The numbers of bacteria varied tremendously on the individual leaves of the same sample. No significant effect of benomyl on the total quantities of bacteria colonizing the leaves could be observed.

*The effect of benomyl on the composition of the mycoflora.* The initial aim to create a low and a high level of saprophytic colonization by spraying with benomyl was only partly achieved. In 1972 and in 1974 even the population density of the benomyl-sprayed leaves exceeded 6500 propagules/$cm^2$ within two weeks after flowering, a density apparently high enough for antagonistic action. However, the comp osition of the mycoflora was strongly affected (Table 1). In all three years benomyl suppressed the leaf colonization by *Cladosporium* spp. and by 'pink yeasts' to less than 6% of the colonization on the control leaves and *Aureobasidium pullulans* was completely eliminated. The 'white yeasts', however, which are mainly composed of *Cryptococcus* spp.

Table 1. The effect of benomyl on the percentage of the dominant fungi of the total phyllosphere mycoflora of rye 3–4 weeks after flowering.

| Year and treatment | Total mycoflora (propagules × $10^3/cm^2 \pm$ s.d.) | 'white yeasts' (%) | 'pink yeasts' (%) | *A. pullulans* (%) | *Cladosporium* spp. (%) |
|---|---|---|---|---|---|
| 7 July – benomyl | 156 ± 32 | 22 | 65 | 4 | 9 |
| 1972 + benomyl | 34 ± 16** | 93 (94)[1] | 5 (2**) | 0 (0**) | 2 (4**) |
| 6 July – benomyl | 9.2 | 13 | 26 | 28 | 33 |
| 1973 + benomyl | 2.3 | 88 (172) | 4 (3) | 0 (0) | 8 (6) |
| 26 June – benomyl | 98.0 ± 49.2 | 22 | 34 | 39 | 5 |
| 1974 + benomyl | 64.5 ± 50.6 | 100 (303*) | 0 (0**) | 0 (0**) | 0 (0**) |

** $P \leqslant 0.01$, * $P \leqslant 0.05$, levels of significance were calculated for the total mycoflora and for the absolute figures of each fungal group in 1972 and 1974.
[1] In parentheses: population densities of the particular fungal group as % of its density on the control leaves.

*Tabel 1. Het effect van benomyl op het percentage dominante schimmels van de totale fyllosfeer mycoflora van rogge 3–4 weken na de bloei.*

(Fokkema, 1971), were not affected at all by benomyl. In 1972, their number was about the same on benomyl-sprayed leaves and on the control leaves. In 1973 and in 1974, however, the 'white yeasts' increased to 1.7 and 3 times the population density of the control leaves, respectively.

The differential effect of benomyl on the phyllosphere fungi in the field was comparable to the effect of benomyl to these fungi on agar. The sensitivity to benomyl of at least 10 isolates of each group of saprophytes was tested on PDA plates supplemented with benlate. Growth of isolates of *Aureobasidium pullulans* and *Cladosporium* spp. was reduced by 50% at benomyl concentrations between 0.1 and 1 ppm (active ingredient). Similar growth reductions of isolates of the 'pink yeasts' and 'white yeasts' were obtained at concentrations between 1 and 10 ppm and 10 and 100 ppm, respectively. In accordance with Bollen and Fuchs (1970) and Greenaway (1973) *Cochliobolus sativus* was rather insensitive to benomyl. Its growth was reduced by 50% at benomyl concentrations between 10 and 100 ppm.

## Discussion

The capacity of the natural saprophytic mycoflora to reduce infection of rye by *C. sativus* could be demonstrated just after flowering, provided that the population density on the control leaves was at least 3000 propagules/$cm^2$ and that benomyl treatment had reduced this to less than 25%. When the benomyl-sprayed leaves also reached population levels of 6500 propagules/$cm^2$ or more, no correlation could be found between the density of the mycoflora and its buffering capacity against infection by *Cochliobolus sativus*. Bacteria did not seem to be involved in the buffering effect of the phyllosphere microflora.

Although the results in the replicate experiments varied considerably, they can be

explained and are in accordance with those of previous glasshouse experiments (Fokkema, 1973). In the field experiments the density of saprophytic colonization was an uncertain factor. It was assumed to be stimulated by the natural pollen deposit, and on the other hand a considerable reduction of the colonization was expected by fungicidal treatment.

Inoculation too close to flowering time could imply that also the control leaves were not yet sufficiently colonized, as on 14 June 1974. Besides, in this particular year, the rainfall during flowering may have washed off a part of the pollen deposit, (see also Fokkema, 1971). With inoculation longer after flowering, even the mycoflora on the benomyl-sprayed leaves reached a sufficient antagonistic level due to the tolerance of the 'white yeasts' to the fungicide, as shown on 26 June 1972 and 21 June 1974. The population densities after flowering in 1973 were relatively low. An explanation may be the high temperatures, reached on the days before sampling. Air temperatures of almost 30°C have been found to reduce the colonization by the yeasts (Fokkema, 1971; Warren, 1972a). Therefore the colonization before the warm period might have been higher than the data suggest. Since the mechanism of this antagonistic action is probably competition for nutrients released from the pollen (Fokkema, 1973), the microbial activity between flowering and inoculation has more influence on the antagonistic capacity than the density of the mycoflora at the moment of inoculation.

Knowledge of the concentrations of the saprophytes necessary for antagonistic action is mainly based on 'mixed-inoculation' experiments in the glasshouse. Warren (1972b) obtained 50% reduction in the number of expanding *Phoma betae* lesions on sugarbeet when the concentration of the added yeasts was 2000 cells/cm$^2$, but in presence of pollen 100000 cells/cm$^2$ were needed. On rye, in presence of pollen, yeast densities varying from 30000 to 600000 cells/cm$^2$ did not result in differential antagonistic action against *C. sativus* (Fokkema, 1973), which is in accordance with our field experiments. Yeast populations varying from 12500 to 225000 cells/cm$^2$ reduced, independently of their density, *Alternaria porri* infection of onion leaves by c. 50% (Fokkema and Lorbeer, 1974). In contrast, Van den Heuvel (1969, 1970) reports increasing antagonism against *Alternaria zinniae* on bean leaves at increasing concentrations of *Alternaria tenuissima* and *Aureobasidium pullulans* from c. 6000 to 60000 conidia/cm$^2$ and from 2–20 $\times$ 10$^6$ cells/cm$^2$, respectively. It seems impossible to predict which density of phyllosphere fungi might be sufficiently antagonistic. Factors as the inoculum density of the pathogen (Warren, 1972b), the nutrients in the phyllosphere, the equal distribution of the antagonists over the leaf surface, and environmental factors which affect the working distance of antagonism by a single cell or colony, determine the final antagonistic effect. In the field, the steady development of the saprophytic leaf mycoflora creates a phyllosphere which is unfavourable for the perthotrophic pathogen before it even arrives. This may lower the minimum concentration necessary for antagonistic action in the field. From our experience it is likely that in the field no antagonism by phyllosphere fungi occurs if the population densities are below 3000 propagules/cm$^2$. Phyllosphere populations of 10000 propagules/cm$^2$ and above may have interesting antagonistic properties. With the method used, however, there may be some underestimation of the population densities due to propagules remaining on the leaves even after vigorous washing (Warren, 1972a).

The harmful effect of especially wide-spectrum fungicides on the buffering capacity of saprophytic fungi has been discussed, since it was found that fungicides affected

the saprophytic colonization (Hislop and Cox, 1969; McKenzie, 1971; Dickinson, 1973; Jenkyn and Prew, 1973; Gross and Kenneth, 1973; Warren, 1974). Frahm (1973) reviewed several reports concerning increased infection after benomyl treatment by pathogens tolerant to this fungicide. One would expect that a tolerant pathogen should not be affected by benomyl, but does this automatically imply a stimulation? The 'white yeasts' were not stimulated by benomyl in 1972, and only moderately in 1973 and in 1974. In case of increased infection of *C. sativus* after benomyl treatment it is more likely that damage to the naturally occurring antagonism is involved as suggested by Van der Hoeven and Bollen (1972). Our investigation clearly demonstrated that if benomyl-sprayed leaves show heavier infection than the control leaves, this is due to the reduction of the saprophytic mycoflora to a level which is no longer antagonistic. In the case of benomyl the 'white yeasts' very soon took over the buffering role of the whole mycoflora. When using fungicides it is necessary to be aware of the susceptibility of naturally occurring biological control mechanisms which may come to light only after disturbance of the biological balance. This may be particularly serious in anemophilous crops where the saprophytes play a role as scavengers by consuming the nutrients released from pollen.

More detailed investigation on the possibility of raising an already present mycoflora to an antagonistic level and on the possible side effects of such a mycoflora by promoting leaf senescence is necessary to make applied biological control more promising.

**Samenvatting**

*De bufferende werking van de natuurlijke mycoflora van roggebladeren tegen infectie door Cochliobolus sativus, en de verstoring hiervan door benomyl*

In veldproeven werden roggebladeren, voor de bloei en op verschillende tijdstippen na de bloei, geïnoculeerd met *Cochliobolus sativus* (*Helminthosporium sativum*) teneinde het antagonisme tegen dit pathogeen door de van nature voorkomende schimmelflora – inclusief gisten – te bestuderen. Eerder uitgevoerde kasproeven hebben aangetoond dat saprofytische fyllosfeerschimmels met succes met perthotrofe pathogene schimmels wedijveren om de voedingsstoffen die vrijkomen uit het stuifmeel (Warren, 1972b; Fokkema, 1973).

Verschillende niveaus van saprofytische bladkolonisatie werden verkregen door de proefveldjes met benomyl of met water te behandelen (Fig. 1). *C. sativus* is relatief weinig gevoelig voor benomyl. Het gemiddeld percentage necrotisch bladoppervlak ten gevolge van inoculatie met *C. sativus* vlak na de bloei was in 1972 en in 1973 ruim 60% lager op met water behandelde bladeren dan op met benomyl behandelde bladeren (Fig. 2). Op dit moment heeft de natuurlijke schimmelflora op de met water bespoten bladeren een populatiedichtheid bereikt van 10000 propagula per $cm^2$ bladoppervlak in 1972 en van 3000 propagula per $cm^2$ in 1973. Benomylbespuiting beperkte de kolonisatie tot 1200 en 400 propagula per $cm^2$ in respectievelijk 1972 en 1973, hetgeen blijkbaar een reductie van de antagonistische capaciteit van de schimmelflora tot gevolg had. In 1974 bereikte de saprofytische kolonisatie op de met water behandelde bladeren, vlak na de bloei, slechts een niveau van 500 propagula per $cm^2$, hetgeen niet genoeg bleek om antagonistisch te kunnen zijn.

Benomyl had een gedifferentieerd effect op de aantallen fyllosfeerschimmels: *Cryptococcus* spp. ('witte gisten') werden niet nadelig beïnvloed, *Sporobolomyces roseus* ('rode gisten') en *Cladosporium* spp. werden gereduceerd tot minder dan 6% van hun populaties op de contrôle bladeren, en *Aureobasidium pullulans* werd geëlimineerd (Tabel 1). De bacterieflora varieerde enorm per blad en werd niet door benomyl beinvloed.

Wanneer later in het seizoen in 1972 en in 1974 de 'witte gisten' op de met benomyl bespoten bladeren populatiedichtheden bereikten van 6500 propagula/cm$^2$ of meer, dan was de infectie van deze bladeren vergelijkbaar met die van de met water behandelde bladeren met hogere populatiedichtheden (Fig. 2). Boven een populatiedichtheid van 6500 propagula/cm$^2$ bestond er geen correlatie meer tussen de dichtheid van de antagonisten en hun werking.

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