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Use of carbendazim and carbendazim-resistant yeasts to create different yeast densities on wheat leaves for field studies on biological control

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Abstract

Phyllosphere yeasts may play an important role in suppressing necrotrophic pathogens. In a randomized block design, yeast densities on flag leaves and second leaves of field-grown wheat were left unchanged (D), or were lowered by sprayings with carbendazim (A), or were raised by sprayings with a mixture of *Sporobolomyces roseus*, *Cryptococcus laurentii* var. *flavescens* and nutrients (B), or with carbendazim, carbendazim-resistant *S. roseus* and *C. laurentii* var. *flavescens* and nutrients (C). The application of carbendazim to plots A and C would avoid a biased interference of carbendazim due to other effects than reducing the natural yeast population. Prolonged differences in yeast density would be reflected in differences in severity of diseases caused by carbendazim-insensitive necrotrophic pathogens.

Both treatments B and C enhanced the yeast density about 10-fold at the beginning of leaf colonization, 2 to 3 weeks after leaf emergence. Untreated leaves (D), however, were rapidly colonized by naturally occurring yeasts, reducing the difference between yeast-treated and untreated leaves. Initially, the yeasts were suppressed by carbendazim (A). However, progressively the yeast population consisted of carbendazim-resistant strains which made the carbendazim sprayings ineffective. Therefore, the substantial differences in yeast density were of limited duration. The effect of the treatments on naturally occurring *Mycosphaerella graminicola* (anam. *Septoria tritici*), *Puccinia recondita* f. sp. *tritici* and the total necrotic leaf area (D-value) is discussed.

Additional keywords: Cryptococcus laurentii var. *flavescens*, fungicide resistance, *Mycosphaerella graminicola*, phyllosphere, *Puccinia recondita* f. sp. *tritici*, *Sporobolomyces roseus*.

Introduction

Necrotrophic leaf pathogens of cereals, such as *Septoria nodorum* and *Cochliobolus sativus*, are sensitive to antagonism by the common phyllosphere yeasts *Sporobolomyces* spp. and *Cryptococcus* spp. (Blakeman and Fokkema, 1982). This has been demonstrated under controlled climate cabinet conditions (Fokkema, 1973; Fokkema and Van der Meulen, 1976; Bashi and Fokkema, 1977; Fokkema et al., 1983) and in experiments on field-grown wheat leaves (Fokkema et al., 1975; Fokkema et

al., 1979). In these experiments in which yeasts reduced the degree of leaf necrosis after artificial infection with the pathogen, no information on the effect of yeasts on natural disease development was obtained.

In order to demonstrate the agricultural importance of naturally occurring yeasts in moderating disease development in the field, it is necessary to create prolonged and substantial differences in the population densities of yeasts in the phyllosphere. This may be achieved by (*a*) reducing the natural yeast population by fungicides or (b) stimulating growth of the yeast population by spraying with yeasts and nutrients. Using method *a*, a long-lasting effect can be achieved, but antagonism can only be demonstrated for pathogens not sensitive to the fungicide used. Following method *b*, the yeast density on the treated leaves will only differ substantially from the control leaves at the beginning of leaf colonization, as this difference will fade away rapidly due to the development of natural colonizers (Fokkema et al., 1979). Method *a* has the disadvantage, particularly when yields are to be compared, that the fungicide will not only affect the yeasts but also a number of naturally occurring pathogens. In addition, some fungicides may have a direct effect on the plant, which may complicate the interpretation of the results (Fokkema, 1981).

These disadvantages might be overcome by a method (*c*) by which plants to be treated with yeasts receive the fungicide treatment in combination with the application of fungicide-resistant yeasts and nutrients. In this paper the applicability of method *c* on wheat is described using the fungicide carbendazim which was expected to control the yeasts (Fokkema et al., 1979; Fokkema and De Nooij, 1981) but not to affect *Fusarium* and *Alternaria* spp. In previous years wheat flag leaves and ears had seriously been attacked by *Monographella nivalis* (anam. *Gerlachia nivalis*). This pathogen is difficult to control chemically, but might be controlled by phyllosphere yeasts (Fokkema, 1983).

Materials and methods

Wheat cultivation. The experiments were performed on winter wheat, *Triticum aestivum* L. cv. *Citadel*, at the experimental farm 'De Kandelaar' located at Flevoland, the Netherlands, in 1983. Four treatments were given in a randomized block design with six replicates, eacht individual plot measuring 50 m^2.

Treatments. Yeast densities were assessed after the following treatments:
A. treatment with carbendazim (Delsene I.C.I., 45% a.i., 550 ml ha^{-1}),
B. treatment with yeasts and nutrients (whey powder, 0.5%),
C. treatment with carbendazim, carbendazim-resistant yeasts and nutrients,
D. control (no treatment).

Treatments were applied with a propane operated field plot sprayer (8 l/100 m^2 on 27 May (fungicides only) or 31 May (yeasts and nutrients only) and on 9 June, 15 June, 22 June and 4 July (all treatments).

The yeasts consisted of a mixture of the pink yeast, *Sporobolomyces roseus*, and the white yeast, *Cryptococcus laurentii* var. *flavescens* (Fokkema, 1973).

Colonies of carbendazim-resistant mutants of *S. roseus* had been selected after plating 100 μl suspension of *S. roseus* (10^8 cells ml^{-1}) on potato dextrose agar (PDA) with 2 μg carbendazim (Bavistin, BASF 50% a.i.) per ml. Mutagenesis was induced by ex-

posing the plates to UV irridation (50 W, distance 50 cm) for 1 minute immediately after plating. The few colonies appearing on agar were suspended in 3 ml sterile water, and 100 μl of this was plated on agar containing 400 μg carbendazim ml^{-1}. A resistant strain of *S. roseus* was selected from these plates for further experimentation. A carbendazim-resistant strain of *C. laurentii* var. *flavescens* was selected in a similar way.

Yeast cultivation and inoculum production. The wild-types of *S. roseus* and *C. laurentii* var. *flavescens* were grown on PDA slants at 23 °C. The carbendazim-resistant strains were alternately cultured on agar slants containing 100 or 1000 μg carbendazim ml^{-1}. Cold storage of the resistant strains was avoided because occasionally the resistant strains did not survive 4 °C. A similar phenomenon has been discussed by Andrews (1986).

Yeasts were grown separately in shake culture at 20 °C in 1000-ml Erlenmeyer flasks with 300 ml nutrient solution containing 0.1% yeast extract, 1% bactopeptone and 83 mM D-glucose (1.5%). For culturing carbendazim-resistant yeasts 200 μg carbendazim ml^{-1} was added. Six to eight days after addition of 3 ml of inoculum (c. 10^8 cells ml^{-1}) obtained from the agar slants; the yeast concentration in the cultures amounted to 3-9 $\times$ 10^8 cells ml^{-1}. The suspensions were diluted in the field with water containing 0.05% Tween 80 and 0.5% whey powder as a nutrient. The final concentration of each yeast strain in the spraying solution was c. 2 $\times$ 10^7 cells ml^{-1}.

Analysis of the phyllosphere mycoflora. At intervals of 6-13 days, six flag leaves and six second leaves were collected from each individual plot. After measurement of the leaf areas, each sample consisting of pieces of six leaves was shaken vigorously in 200 ml 0.01% Tween 80 for 1 h. After appropriate dilution, 100 μl of the washing liquid containing yeast cells was plated in triplicate on basal yeast agar (BYA) plates (Fokkema et al., 1979). In addition, leaf washings from carbendazim-treated (A and C) and untreated (D) plots were plated on BYA agar supplemented with 20 μg carbendazim ml^{-1} for determining densities of carbendazim-resistant yeasts. For appropriate comparison, however, all total yeast numbers (Fig. 1) are based on colony counts from BYA without carbendazim. Colonization was expressed as the number of colony forming units (CFU) cm^{-2} (upper and lower) leaf surface. When sampling dates coincided with spraying dates, sampling was performed first.

Disease assessment. Per plot, 15 culms were collected at random for disease assessment. Infection by *Mycosphaerella graminicola* (anam. *Septoria tritici*) was determined by estimating the percentage necrotic leaf area with pycnidia. Leaf rust, *Puccinia recondita* f. sp. *triticii*, was assessed by the number of sori per leaf. Further, the percentage dead leaf area (D-value) which could not be attributed to a specific disease was estimated. Leaf and ear infection by *M. nivalis* did not occur.

Results

Yeast population dynamics. The population densities of the pink and white yeasts for all treatments are calculated from the number of colonies appearing on BYA plates without carbendazim. Therefore the colonization figures potentially consist of carbendazim-sensitive as well as carbendazim-resistant CFU.

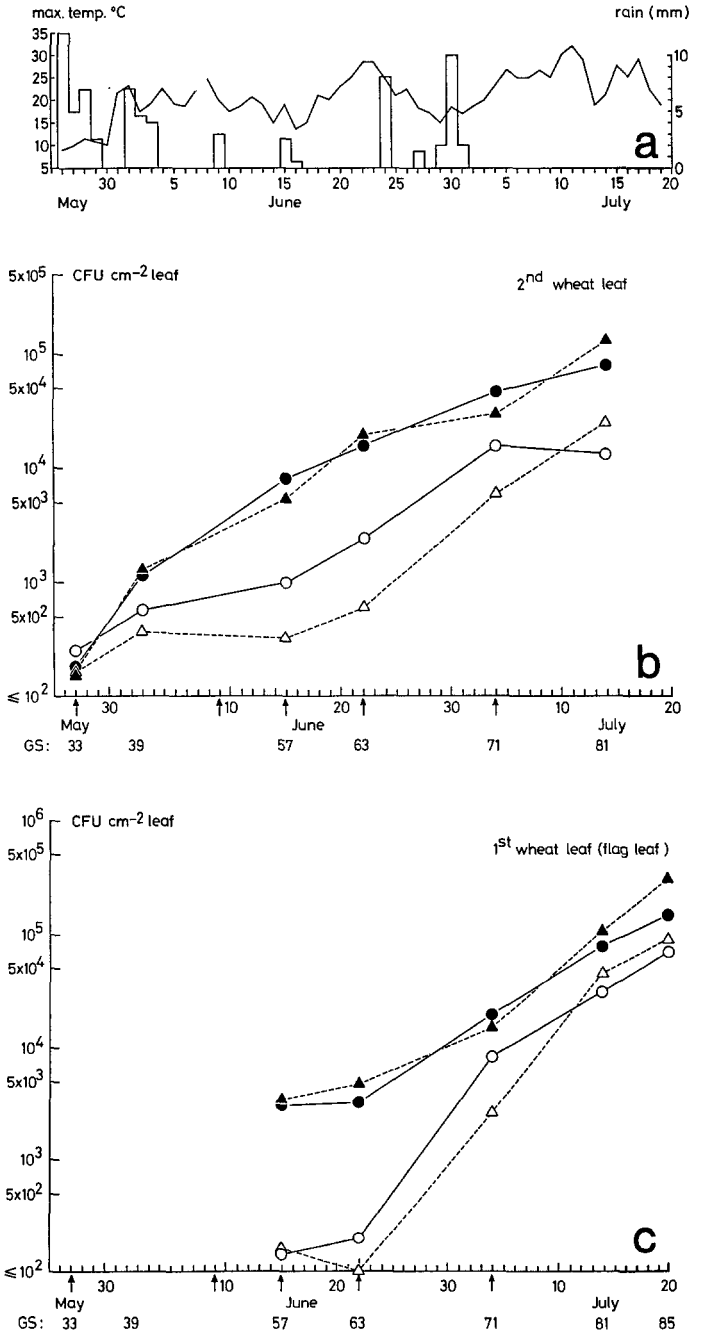

Fig.1. Effect of different treatments on the seasonal development of pink and white yeasts on second (penultimate) leaves (b) and flag leaves (c) of wheat in relation to rainfall and maximum air temperature (a). The treatments consisted of A: carbendazim (▵), B: yeasts and nutrients (•), C: carbendazim, carbendazim-resistant yeasts and nutrients (▲), D: untreated (○). Arrows indicate the spraying dates; GS: decimal code for the growth stage (Zadoks et al., 1974).

The following effects of the treatments on the seasonal development of yeasts on the second leaf and the flag leaf (leaf 1) can be recognized (Fig. 1). Spraying with yeasts and nutrients increased the population density 5- to 10-fold, irrespective of fungicide resistance of the yeasts applied. However, the differences in population density became less substantial at the end of the season, particularly on the flag leaves, because of the rapid development of the native yeasts on the untreated leaves. Carbendazim significantly reduced the native yeast population on leaf 2 on several sampling dates (15, 22 June and 4 July), but on leaf 1 a significant reduction was only detected on 4 July ($P \leqslant 0.05$, Wilcoxon's two sample test).

Composition of the yeast population. Based on colony colour, cereal phyllosphere yeasts can be divided in two distinct groups, viz. the pink yeasts and the white yeasts, which consist mainly of *Sporobolomyces* spp. and *Cryptococcus* spp., respectively (Fokkema, 1973). The effect of the treatments on the occurrence of these groups is presented in Tables 1 and 2, together with the percentage carbendazim resistance within these groups.

On the untreated control plots (D) the pink yeasts generally predominated on the top two leaf layers. The figures also show that the non-yeast part of the saprophytic mycoflora, which mainly consisted of *Cladosporium* spp. and to a lesser extent of *Aureobasidium pullulans*, was insignificant with the exception of senescing second leaves on 14 July. Treatment with yeasts (wild-types) and nutrients (B) initially resulted in more pink yeasts than white yeasts; however, from 4 July onwards, white yeasts became slightly dominant. More or less equal numbers of white and pink yeasts were found on leaves sprayed with carbendazim (A). The white yeasts usually outnumbered the pink yeasts on leaves treated with carbendazim-resistant pink and white yeasts,

Table 1. Effects of different treatments on the relative occurrence (%) of pink (PY) and white (WY) yeasts of the total saprophytic mycoflora of penultimate wheat leaves (leaf 2) and on the percentage carbendazim resistance (r.%) in these populations during leaf colonization.

Treatment	Yeast type	Sampling date									
		2 June		15 June		22 June		4 July		14 July	
		%	r.%	%	r.%	%	r.%	%	r.%	%	r.%
A. carbendazim	PY	66	10	59	100	33	50	43	100	55	100
	WY	18	37	35	100	67	50	53	100	41	100
B. yeasts + nutrients	PY	71	—[1]	54	—	54	—	43	—	44	—
	WY	28	—	46	—	46	—	56	—	54	—
C. carbendazim + resistant yeasts + nutrients	PY	66	100	24	100	26	100	14	100	19	100
	WY	28	100	74	100	74	100	85	100	80	100
D. untreated	PY	77	2	90	—	75	6	64	—	49	8
	WY	21	0	9	—	25	17	35	—	15	73

[1] —: not determined.

Table 2. Effects of different treatments on the relative occurrence (%) of pink (PY) and white (WY) yeasts of the total saprophytic mycoflora of wheat flag leaves (leaf 1) and on the percentage carbendazim resistance in these populations (r.%) during leaf colonization.

Treatment	Yeast type	Sampling date							
		15 June		22 June		4 July		14 July	
		%	r.%	%	r.%	%	r.%	%	r.%
A. carbendazim	PY	82	72	—[1]	—	63	53	49	66
	WY	12	50	—	—	37	50	49	81
B. yeasts + nutrients	PY	64	—	70	—	43	—	42	—
	WY	35	—	30	—	56	—	57	—
C. carbendazim + resistant yeasts + nutrients	PY	26	94	54	96	16	64	14	84
	WY	72	98	46	100	82	100	85	100
D. untreated	PY	80	—	50	0	76	—	70	16
	WY	13	—	50	0	24	—	27	39

[1] —: not determined.

carbendazim and nutrients (C). Apparently the competitive ability of the introduced pink and white yeasts differed from that of the corresponding naturally occurring yeast populations.

Carbendazim-resistant pink and white yeasts were present among the indigenous mycoflora of leaves from the control plots (D). The percentage carbendazim-resistant pink and white yeasts on the second leaf layer of the untreated plants increased during the season reaching 8 and 73%, respectively, on 14 July (Table 1). On the untreated flag leaves 16% of the pink yeasts and 39% of the white yeasts were carbendazim-resistant on 14 July (Table 2).

Spraying with carbendazim increased the relative occurrence of carbendazim-resistant pink and white yeasts dramatically. This explains why carbendazim had little effect on the total yeast population.

Where carbendazim-resistant yeasts were applied to the leaves, the percentage resistance was high throughout the period of sampling. It is not known whether the resistant yeast population was composed of the introduced strains or of naturally occurring resistant yeasts. However, the difference between the ratio of pink and white yeasts on leaves treated with carbendazim-resistant yeasts (C) and that on leaves treated with carbendazim alone (A) suggests a significant contribution of the introduced yeasts (Table 1 and 2).

Yeasts and disease development. Weather conditions largely governed disease development. During May maximum daily temperature remained below 15 °C, and 24 days with rainfall were recorded. This favoured the development of speckled leaf blotch (*M. graminicola*). During June and July, the weather was mainly warm and dry (Fig. 1) and was therefore unfavourable for *M. nivalis*. From mid June *P. recon-*

Table 3. Effect of different treatments on the occurrence of speckled leaf blotch (M.g., % necrotic leaf area) and leaf rust (P.r., sori leaf^{-1}) and on the total dead leaf area (D-value, %) on field grown wheat. Mean values are from leaf 2 on 4 July and leaf 1 on 14 July.

Treatment	Number of plots[1]	Sampling date					
		4 July			14 July		
		M.g.	P.r.	D-value	M.g.	P.r.	D-value[2]
A. carbendazim	6	3.8a[3]	16a	0.6a	1.5ac	114a	51a
B. yeasts + nutrients	6	7.6b	22a	4.9b	4.4ab	131a	75b
C. carbendazim + resistant yeasts + nutrients	6	3.0a	11a	0.1a	1.1c	128a	42a
D. untreated	12	8.3b	9a	12.2c	4.5b	174a	82b

[1] Sample size per plot: 15 culms.
[2] Relating to leaf 2.
[3] Values within each column not followed by the same letter are significantly different at $P < 0.05$ (ANOVA followed by the T-method for multiple comparisons (Sokal and Rohlf, 1981)).

dita f. sp. *tritici* developed rapidly. Because of these dry weather conditions, ripening diseases on flag leaves and ears were almost absent.

On 4 July (leaf 2) and on 14 July (leaf 1) speckled leaf blotch (Table 3) was significantly reduced by carbendazim, irrespective of the addition of yeasts. Leaf rust infection (Table 3) on both sampling dates varied with the treatments, but the differences were not significant nor consistent. The percentage dead leaf area (D-value, Table 3) was markedly less in plots sprayed with carbendazim ($P \leqslant 0.05$). Also the sprayings with yeasts resulted in a moderate, but significant reduction of the D-value on 4 July.

Discussion

The field experiment described has been carried out to test an experimental design (Fokkema, 1983) for evaluating biological control of necrotrophic pathogens by naturally occurring yeasts under agricultural conditions. The creation of prolonged and substantial differences in the population densities of the phyllosphere yeasts was emphasized, with special care that the means used for creating such a difference excluded interference by direct effects of fungicides on pathogens or plants. It was anticipated that the treatment with carbendazim on one hand and spraying with carbendazim, carbendazim-resistant yeasts and nutrients on the other hand would result in the desired difference. The difference actually achieved, however, was hardly larger than that obtained in a classic way, by just spraying wheat leaves with yeasts plus nutrients (Fokkema et al., 1979). This was mainly due to the failure of carbendazim to sufficiently suppress the yeast populations, because of the rapid development of naturally occurring carbendazim-resistant yeasts. Platings of leaf washings from second leaves sprayed with carbendazim often resulted in even more CFU on BYA with carbendazim than

on BYA without carbendazim. This phenomenon may indicate a certain dependency of the carbendazim-resistant yeasts on carbendazim or a general stimulation of colony development by carbendazim.

Previous studies have reported substantial reductions of populations of pink yeasts and of the total yeasts on cereal leaves following sprayings with benzimidazole fungicides (Jenkyn and Prew, 1973; Fokkema et al., 1975; Dickinson and Wallace, 1976). The long-standing application of these fungicides in cereals has apparently resulted in the establishment of carbendazim-resistant strains in the population. Furthermore, in the untreated plots, the percentage of carbendazim-resistant yeasts rose during the season, which is difficult to explain. It is unlikely that the carbendazim-resistant yeasts originated from the plots treated with these yeasts (C) because of the size of the plots relative to the likely horizontal dispersal of *S. roseus*. It was observed (Fokkema, unpublished results) that the percentage carbendazim-resistant *S. roseus* CFU (4%) did not change at distances of 3 to 10 m from a plot where carbendazim-resistant *S. roseus* had been introduced. Moreover, in 1987 the percentage carbendazim-resistant *S. roseus* CFU amounted to c. 15% in an unsprayed wheat field (Dik, unpublished results).

Considering the natural occurrence of carbendazim-resistant *S. roseus*, it is important to realize that the use of fungicide resistance as a means to mark micro-organisms in dispersal studies (Andrews, 1986) might be inadequate in agricultural environments where this fungicide has previously been used.

Rainfall was recorded continuously, and unfortunately spraying dates (Fig. 1) were often followed by days with rain. This may have washed away some of the added yeasts, nutrients and fungicides. Still, the moderate effect of the latter seems primarily due to the development of carbendazim-resistant strains.

Phyllosphere yeasts have proved to be able to reduce infection of various necrotrophic but not of biotrophic pathogens (Blakeman and Fokkema, 1982). This general difference in response, which seems inherent to the pathogen's sensitivity to nutrient competition (Fokkema, 1981), explains why the yeasts in our field experiment did not reduce the development of leaf rust. Our observations on 4 July (Table 3) suggest a stimulation of rust infection of leaves treated with yeasts and nutrients or with carbendazim. However, this was not significant and moreover a combination of these treatments had not such an effect. On the other hand, Parker and Blakeman (1984) observed that *Cryptococcus* spp. stimulated germ tube growth of *Uromyces viciae-fabae* and subsequent infection on detached broad bean leaves. This interesting phenomenon may not be applicable to *P. recondita* because previous glasshouse experiments could not demonstrate an effect of phyllosphere yeasts on the infection of rye by *P. recondita* f. sp. *recondita* (Warren, 1972; Fokkema, unpublished results).

Apart from the basic ability of yeasts to reduce infection of necrotrophic pathogens, a certain threshold density of yeasts is needed for a measurable antagonistic effect. For example on cereal leaves a minimum density of c. 10 000 yeast cells cm^{-2} may be required for a substantial reduction of infection by *Septoria nodorum* or *Cochliobolus sativus* (Fokkema et al., 1975; Fokkema et al., 1979). In our experiment enhancement of the yeast density (treatment B) did not reduce the infection by *M. graminicola*, although it could be expected that this necrotrophic pathogen, like *S. nodorum* (Fokkema and Van der Meulen, 1976) would be sensitive to antagonism by yeasts. The absence of such an effect is very likely to be caused by insufficient numbers of yeasts colonizing the leaves at the time of possible interaction, i.e. during the disease-

promoting weather conditions in May and the first week of June (Shaner and Finney, 1976). Due to the relatively long latent period of *M. graminicola* (Brokenshire, 1976), the necrotic areas with pycnidia assessed on 4 July (2nd leaves) and on 14 July (flag leaves) may have originated from penetrations several weeks earlier. The critical level of 10 000 yeast cells cm^{-2} on the second and flag leaves was only reached on 22 June and on 4 July, respectively. By this time, weather conditions had become much less favourable for infection by *M. graminicola*. Moreover, particularly on the flag leaves, the differences in yeast densities between leaves treated with yeasts (B) and untreated leaves (D) had reduced since the number of yeast cells on the latter was rapidly increasing. The percentage dead leaf tissue (D-value), which was estimated in addition to specific disease symptoms, included natural senescence as well as disease symptoms which could not be distinguished. Apart from the reduction of disease, carbendazim may reduce natural senescence by its cytokinin-like properties (Thomas, 1974). Interestingly, the treatment with yeasts and nutrients significantly reduced the D-value on 4 July from 12 to 5%, which may include reduction of irrecognizable non-sporulating lesions of *M. graminicola* and other pathogens. There is no indication that the treatment with yeasts had any adverse effect on the leaf condition. This is in agreement with previous field experiments in which the effect of phyllosphere saprophytes on wheat yield have been studied (Rabbinge et al., 1984).

The experimental failure in our study to sufficiently suppress the native yeast population by carbendazim may be disappointing, because the approach followed looked suitable for the appropriate evaluation of the antagonistic potential of naturally occurring phyllosphere yeasts under field conditions. Nevertheless, there is sufficient circumstantial evidence that yeasts have a moderating effect on necrotrophic pathogens (Blakeman and Fokkema, 1982), particularly on flag leaves and ears of cereals where naturally high yeast densities occur. It seems worthwhile to select fungicides carefully in order not to eliminate the yeasts (Fokkema, 1987) and to take advantage of this naturally occurring biological control system.

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Samenvatting

Het gebruik van carbendazim en carbendazim-resistente gisten om verschillende dichtheden van gisten op tarwebladeren te realiseren ten behoeve van biologische bestrijdingsproeven in het veld

Fyllosfeergisten kunnen misschien een belangrijke rol spelen in de onderdrukking van necrotrofe pathogenen. In een blokkenproef werd de dichtheid van gisten op vlagbladeren en tweede bladeren niet beïnvloed (D) of verlaagd door bespuitingen met carbendazim (A), of verhoogd door bespuitingen met een mengsel van gisten (*Sporobolomyces roseus* en *Cryptococcus laurentii* var. *flavescens* en voedingsstoffen (B), of met een mengsel van carbendazim, carbendazim-resistente *S. roseus* en *C. laurentii* var. *flavescens* en voedingsstoffen (C). De grootste verschillen in dichtheden van de

gistpopulaties werden verwacht tussen de behandelingen A en B of C. Door alle veldjes A en C met carbendazim te behandelen wordt een eenzijdig effect van carbendazim, berustend op andere eigenschappen dan de onderdrukking van de natuurlijke gistpopulatie, voorkomen. Langdurige verschillen in de populatiedichtheden van de gisten zouden weerspiegeld kunnen worden in verschillen in aantasting door carbendazim-ongevoelige necrotrofe pathogenen.

Aan het begin van de bladkolonisatie door gisten, 2 tot 3 weken na het verschijnen van het blad, leidde zowel behandeling B als C tot een 10-voudige verhoging van de gistdichtheid. De onbehandelde bladeren (D) werden echter spoedig door de van nature voorkomende gisten gekoloniseerd en het verschil tussen de onbehandelde en met gist bespoten bladeren werd snel kleiner. Aanvankelijk werden de gisten door carbendazim (A) onderdrukt. Geleidelijk bestond de gistpopulatie echter uit carbendazim-resistente stammen, zodat de carbendazim bespuitingen nauwelijks meer effect hadden. Hierdoor werden grote verschillen in gistdichtheden niet langdurig gerealiseerd. Het effect van de behandelingen op de natuurlijke infectie door *Mycosphaerella graminicola* (anam. *Septoria tritici*), *Puccina recondita* f. sp. *tritici* en op het totale dode bladoppervlak (D-waarde) wordt besproken.

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Book review

R.A.C. Jones & L. Torrance (Eds), 1986. Developments and applications in virus testing. Developments of Applied Biology. 1. Association of Applied Biologists (AAB), Wellesbourne, England. 312 pp, 61 figures, 29 tables. Price hardback: £ 30.00 (£ 24.00 for AAB members).

The current interest in techniques to detect viruses and viroids and the enormous scope for their application as research tools and in practical routine testing stimulated the AAB Virology Group to host an international conference. The resulting book contains 15 chapters selected from the conference presentations. It also includes three invited chapters. Because of his outstanding contribution to plant virology, it is dedicated to the memory of B. Kassanis, who died early in 1985.