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BACTERIAL ANTAGONISM IN SEED HEALTH TESTS[1,2]

De invloed van bacterieel antagonisme op de resultaten van gezondheids-onderzoek van zaaizaad

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DE TEMPE & LIMONARD (1965) reported a suppressing effect of high blotter moisture in seed health testing for a number of seed-borne fungal pathogens. The present author proved this to be due to bacterial antagonism, among others by adding antibacterial antibiotics to the blotters.

INTRODUCTION

It is a well-known fact in seed-testing routine that changes in the environment influence the percentage of pathogenic fungi found for a number of seed-borne diseases. It was reported by DE TEMPE & LIMONARD (1966) that this is the case with changes in the substrate moisture level in the blotter method. The suppressing effect of high blotter moisture was first observed by them with *Botrytis cinerea* infection of flax-seed. Similar results were obtained with some other seed-borne infections (*Phoma exigua* var. *linicola* in flax-seed, *Stemphylium radicinum* and *Alternaria porri* f. sp. *dauci* in carrot seed, *Alternaria brassicicola* in cabbage seed and *Alternaria raphani* in radish seed) but not with some other ones (*Fusarium* spp. and *Helminthosporium* spp. in cereal seeds and *Phoma betea* in beet seeds). For the sake of convenience the above phenomenon will be indicated as the "wet blotter effect" or WBE.

This publication deals with an investigation on the background of this phenomenon. For this purpose the author used mainly *B. cinerea* infection of flax-seed wich was readily available to him. The results of blotter testing for this seed-infection are known to be lower than those obtained by the agar method (DE TEMPE, 1958, 1961, 1963; VAN DER SPEK, 1965). The reason for this latter phenomenon was also studied and turned out to be related to the WBE.

MATERIALS AND METHODS

Unless stated otherwise, the experiments were carried out as follows.

Blotter method

Blotter tests were normally made on white blotter papers of 10 × 25 cm, underneath which a thick grey blotter (coarse paper wadding) was placed to serve as moisture stabilizer. These blotters were put in perforated zinc trays. In "dry" blotter tests the blotters were wetted in a tray with tap water and subsequently pressed against a dry (non-wetted) one. "Wet" blotters were soaked in water to their maximum water holding capacity. This amounted to about 30 ml of water (140% of the blotter weight) in the "dry" and 60 ml of water

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(280% of the blotter weight) in the "wet" blotters. Blotter weight itself was about 22 g (white and grey blotter taken together). In case substances were added to blotters, this was done by soaking the blotters in aqueous solutions of that substance instead of in tap water.

Incubation was carried out in high humidity incubators (above 95% RH), provided with either Philips fluorescent daylight tubes of General Electric near-ultraviolet tubes with a 12/12 hrs cycle of light/darkness. The normal incubation temperature was 20°C. Tests for *B. cinerea* in flax-seed were carried out with daylight tubes, those for *Fusarium* spp. in wheat and *Phoma betae* of beet seeds in the dark and for all others under near-ultraviolet. For each test 400 seeds were used per treatment. In order to prevent secondary spread during the incubation period, 50 seeds were put on a blotter and in the case of *B. cinerea* in flax-seed only 25. For the blotter test for *Fusarium* spp. in wheat one is referred to DE TEMPE (1964).

Agar method

Agar tests were carried out for *B. cinerea* in flax-seed on Oxoid malt extract agar (pH 5.4) in Petri-dishes of 9 cm diameter. Only 5 seeds were put in a Petri-dish, 400 seeds being used per treatment. Inspection was carried out after three and five days. Incubation took place at 20°C in darkness.

Seed moisture determination

Seed moisture determinations were made in duplicate by means of drying in an electric oven at 130°C for 90 minutes according to the routine procedure of the Dutch Government Seed Testing Station as described in its "Methodenboek" (Book of Methods). Wheat seed was ground in a special type of mill before drying. The results are expressed as a percentage of the original weight.

Soaking experiments

Water-soak experiments were carried out by placing small quantities of seed in a 250 ml beaker and covering them with a thin layer of tap water adjusted to the desired temperature. The beakers were then placed in an incubator of that temperature, mostly 20°C during 24 hours. Soaking of the seeds in various solutions took place in an identical way.

pH-Determinations

These were made by means of Panpeha indicator strips obtained from Riedel-De Haën AG Seelze/Hannover.

EXPERIMENTS AND RESULTS

Seed moisture determinations

One of the first questions tackled in this investigation was the effect of blotter moisture upon seed moisture level. This question was thought to be of particular importance as after all the pathogen is present on or in the seed and is thus affected by the latter more directly than by the blotter. To this end different kinds of seeds were incubated on "wet "and "dry" blotters in the same way as for normal seed health tests. After various periods of incubation seeds, were collected to have their moisture content determined. The results of such deter-

minations during the first 24 hours of incubation for seeds of flax, cabbage, wheat and beet, are given in Table 1. In this table the results of a moisture determination for flax seed on Malt extract agar and for wheat seed being soaked in water are also included. The four kinds of seeds mentioned above were chosen because two of them showed WBE (flax with *B. cinerea* and *Phoma exigua* var. *linicola*, and cabbage with *Alternaria brassicicola)* and two did not (wheat with *Fusarium* spp. and *Helminthosporium* spp., and beet with *Phoma betae)*.

TABLE 1. Moisture percentages of seeds of flax, cabbage, wheat and beet, after various periods of incubation on "wet" and "dry" blotters. For flax seed also on malt extract agar and for wheat after soaking in water.

Vochtgehalte als percentage van het oorspronkelijk gewicht voor zaden van vlas, kool tarwe en biet na verschillende incubatietijden op „nat" en „droog" filtreerpapier. Voor vlaszaad ook op moutextractagar en voor tarwe na weken in water.

Hours of incubation	Flax			Cabbage		Wheat			Beet	
	Wet	Dry	Agar	Wet	Dry	Wet	Dry	Water soak	Wet	Dry
0	8.00	8.00	7.60	7.75	7.75	14.50	14.50	14.50	10.60	10.60
1	47.40	20.50	36.30	29.50	18.00	20.90	17.30	21.70	25.85	18.80
2	48.20	29.00	44.20	34.50	23.50	22.50	18.70	25.60	28.10	23.00
3	63.20	37.20	51.40	38.00	28.50	24.10	20.30	28.40	31.00	26.00
4	61.40	40.20	49.60	42.00	34.00	25.10	21.70	30.00	34.35	28.90
5	58.20	40.20	51.20	43.50	37.00	25.90	22.55	31.20	36.50	30.45
6	64.40	46.80	50.50	43.50	37.00	26.40	23.50	32.40	38.35	34.00
15	64.20	54.40	57.90	44.50	42.75	29.90	28.00	38.70	45.80	43.70
24	66.20	61.60	59.90	45.75	44.25	32.80	32.20	41.70	49.25	48.10
Incubatie-tijd in uren	*Nat*	*Droog*	*Agar*	*Nat*	*Droog*	*Nat*	*Droog*	*Ge-weekt in water*	*Nat*	*Droog*
	Vlas			*Kool*		*Tarwe*			*Biet*	

One may note that:

1. The rate of water uptake shows differences between the four kinds of seed. It is the highest for seeds of flax and cabbage, and lower for those of beet and wheat.
2. The water uptake on "wet" blotters is quicker than those for "dry" blotters.
3. The water uptake on malt extract agar in the case of flax seeds is intermediate between that for "dry" and "wet" blotters.
4. The water uptake in the water-soak experiment with wheat is quicker than that on "wet" blotters.
5. The difference in the rate of water uptake on "wet" and "dry" blotters is greater for seeds of flax and cabbage than for those of beet and wheat. It is greatest for flax seed and smallest for wheat seed.
6. The difference in the rate of water uptake on "wet" and "dry" blotters diminishes with time and becomes very small after 24 hours. The small difference then existing remains during the rest of the incubation period.

Is the WBE a property of the seed or of the pathogen?

In the previous section three seed infections on two kinds of smaller seeds and three others on larger seeds were mentioned with regard to WBE. It was interesting to know whether this was due to certain properties of the seed (such as, e.g., rate of water uptake) or of the pathogen. This could be studied with different combinations of the above pathogens and kinds of seeds. Several different observations and small experiments on this problem were made (a few examples of which follow below) which led to the conclusion that the different behaviour of seeds to the WBE does not depend on the pathogen. Natural infections, other than in flax could only be obtained with *B. cinerea* in seeds of lettuce, corn-salad and wheat. In the larger seeds of corn-salad and wheat it showed no WBE, but it did in the small lettuce seeds. Artificially inoculated flax and lettuce seed (obtained by shaking seeds in a Petri-dish with a sporulating culture of *B. cinerea* on MA) showed the WBE very strongly; on "wet" blotters hardly any *Botrytis* was found as contrasted with nearly 100 % on "dry" blotters. However, this kind of artificial inoculation is very superficial and thus makes the WBE perhaps more direct. Sunflower seeds were inoculated while still in the flower head by pouring a spore suspension from agar cultures over it and incubating for two weeks at 20 °C in a polyethylene bag. The seeds were then collected and dried at about 30 °C and 60 % RH in a special seed drying cabinet. The relatively large sunflower seeds were then tested on "wet" and "dry" blotters; a *B. cinerea* percentage of, respectively, 34 and 35 % was found.

Similarly *Fusarium* spp. on small seeds expressed themselves much more frequently and in higher numbers on "dry" blotters than on "wet" ones.

Soaking experiments

The results mentioned before hinted that moisture content of the seed and especially the speed of water uptake were important factors involved in the WBE. One of the next steps was investigating the possibility whether increasing the uptake of non-reacting seeds by special means would lead to a decrease of the percentage of infection found. To this end two wheat samples containing *Fusarium* spp. were soaked for 24 hours in water of 10 °C and of 20 °C and then tested with the blotter method. The *Fusarium* percentages observed were:

Sample	Control	10 °C soak	20 °C soak
1	35 %	32½ %	17¾ %
2	26 %	23¾ %	6¾

It thus appeared that soaking for 24 hours at 20 °C did decrease the percentage *Fusarium* found, while soaking in water of 10 °C did not or hardly. The soaking water of 20 °C had become cloudy and malodorous, whereas that of 10 °C remained clear and without bad odour. This indicated that bacteria might be implicated. To investigate this possibility the same experiment was repeated with one more treatment added in which wheat grains were soaked in a 50 ppm solution of the antibacterial antibiotic terramycin in water. This time the following *Fusarium* percentages were found:

Sample	Control	10°C soak		20°C soak	
		without terramycin	with terramycin	without terramycin	with terramycin
1	33¼%	27¼%	38½%	12¾%	28 %
2	23 %	20½%	26 %	13½%	19½%

These results showed that the presence of terramycin in the soaking water counteracted the "soak effect". Identical results were obtained in soaking beet seeds with *Phoma betae* in water of 20°C with and without terramycin:

Sample	Control	Without terramycin	With terramycin
1	59 %	28¼%	54 %
2	46½%	22 %	41¾%

For both infections as well as for other ones, similar experiments were afterwards carried out using many samples with identical results. In later experiments higher concentrations of terramycin (100 ppm or even more) were used as this proved to give better and more dependable results.

Effect of temperature in the blotter test

The results just mentioned suggested bacterial antagonism as the cause of the WBE. To explore this possibility "wet" and "dry" blotter tests for *B. cinerea* in flax seed were carried out at a temperature of 12°C instead of the usual 20°C in the supposition that at lower temperatures bacterial development would be slower. A temperature of 12°C was chosen as seed germination and seedling development were then still acceptable. The average infections for ten samples were:

	in 8 days	in 11 days
on "wet" blotters	3.30%	5.05%
on "dry" blotters	8.40%	12.18%

The results showed that the WBE was still very clear at 12°C for this infection. The temperature effect as found for the soaking of wheat did apparently not apply here.

Use of antibiotics in blotter tests

To explore the possibility of bacterial antagonism as the cause of the WBE in a different way, antibacterial antibiotics were added to the water serving as blotter moisture. The results are given in Table 2. It should be observed here that the WBE does not only express itself in a reduction of the percentage of in-

fection found, but also in a suppression of the growth of the infections that do appear. On "dry" blotters and on "wet" blotters with antibiotics the growth and spread of *B. cinerea* on the blotters increased markedly.

TABLE 2. The influence of adding antibacterial antibiotics to "wet" blotters on the results of the blotter test for *Botrytis cinerea* in three samples of flax seed.

De invloed van het toevoegen van antibacteriële antibiotica aan „nat" filtreerpapier op het resultaat van de filtreerpapierproef voor B. cinerea *in drie vlaszaadmonsters.*

Testing conditions	Percentage of *B. cinerea* found in sample:		
	No. 1.	No. 2.	No. 3.
"dry" blotter	13½	34½	11½
"wet" blotter	7¾	19¾	6¾
"wet" blotter with 50 ppm terramycin	15	31	12
"wet" blotter with 100 ppm terramycin	10¾	36¼	12¼
"wet" blotter with 100 ppm streptomycin	14	30¾	14¼
"wet" blotter with 50 ppm terramycin and 100 ppm streptomycin	17	34	13¼
Proefomstandigheden	*Percentage* B. cinerea *gevonden in het monster*		

Stimulation of bacterial growth

In order to further consolidate the bacterial antagonism hypothesis for the WBE the following reasoning was used. If suppression of bacterial growth (antibiotics) reduced or eliminated the WBE, stimulation of bacterial growth should increase it. To this end sterile Bacto Nutrient Broth (Difco) was added to the blotters with and without a rough bacterial culture isolated from flax seeds. This reduced the *B. cinerea* percentage to zero in all cases tried. The remarkably strong effect of the sterile broth without bacteria can be explained by stimulation of the growth and development of the natural bacterial seed flora.

Comparison between blotter tests and agar method

The results of seed health testing for *B. cinerea* in flax seed by means of the agar method are higher than those obtained by the blotter method (DE TEMPE, 1958, 1961, 1963; VAN DER SPEK, 1965). This was explained up till now by the fact that the agar test was a more saprophytic test and the blotter test a more pathogenic one. In the latter the fungus was required to attack the young seedling in order to be counted. As the results of "dry" blotter tests came much closer to the results of the agar test than those of "wet" blotters it was now supposed that the reason for the discrepancy between the infection percentages obtained in agar and "wet" blotter tests might be related to the WBE. In order to investigate this possibility somewhat further, 31 flax seed samples were tested in four different ways, viz. on "wet" blotters, on "dry" blotters, on "wet" blotters with 100 ppm terramycin, and on Malt Extract Agar. The four tests were carried out simultaneously for the same sample. The results given in Table 3 confirmed the above-mentioned hypothesis.

TABLE 3. *Botrytis cinerea* percentages found for 31 samples of flax seed tested in four different ways.

Percentage B. cinerea, *gevonden in 31 vlaszaadmonsters, onderzocht op vier verschillende manieren.*

Sample	"Wet" blotters	"Dry" blotters	"Wet" blotters with 100 ppm terramycin	Malt extract agar
1	¼	¾	¾	0
2	0	½	2	½
3	¼	1¼	¾	1
4	0	¾	4¼	1½
5	¼	1¾	2¼	1¾
6	¼	2¼	5	2
7	¾	4	4	2¼
8	½	1	1½	2½
9	½	2	2	2½
10	1½	1½	3¼	3½
11	1¼	6	4	5
12	3	4	6½	5¾
13	3	5	5	6
14	2	2¾	6½	6
15	4¼	4¼	6¼	6½
16	½	5½	6	7
17	2¾	6	8½	7¾
18	2¼	7¾	8¼	8½
19	2	9¼	9	9
20	7¾	12¼	10	10
21	2½	8	9¾	10
22	2½	8½	10¾	10¼
23	6	11½	10¼	10¾
24	2¾	10	13¼	11
25	4	9¾	11¾	11¼
26	3¼	12½	15½	11½
27	6¾	12	15½	12
28	4	14¾	16	15
29	11½	18¾	26¼	21¼
30	22¾	31¼	32	28
31	15¾	27	32¾	30½
Average/ *Gemiddeld*	3.7%	7.8%	9.3%	8.4%
Monster	*„Nat" filtreerpapier*	*„Droog" filtreerpapier*	*„Nat" filtreerpapier met 100 ppm terramycine*	*Moutextractagar*

Influence of pH of substrate

The results just mentioned called forth the question as to why bacterial antagonism does not play a role in the agar test. Two reasons can be thought of. The first is the fact that the agar surface is relatively dry. This point was investigated by means of moisture determinations of flax seed on MA after various periods of incubation, the results of which were already mentioned and given in Table 1. The water uptake of flax seed on MA turned out to be intermediate between that on "wet" and "dry" blotters. "Dryness" of the agar surface could

therefore only give a partial explanation of this problem. The second reason thought of was that the pH of MA might be important. The final pH of the MA-Oxoid that we mostly use is about 5.4, that of MA-Difco 4.6. To compare this with the pH level encountered in blotter testing we checked the pH of blotters during the incubation period by pressing a Panpeha-indicator strip against them. It turned out that the pH of the blotter was 6.9 – 7.0 and remained so during the entire incubation period (pH of the tap water used). It was then decided to change the pH by adding 0.1 M Sörensen phosphate buffer to the blotters in order to increase or lower their pH. The blotters were kept wet during this particular experiment by means of small paper strips hanging in a tray filled with either water or buffer solution. The treatments used were pH 5.2, pH 8.0, tap water (pH 6.9 – 7.0) and "dry" blotters (also pH of tap water). The "dry" blotters were of course without paper wicks. The following average infection percentages were observed:

Testing conditions	*B. cinerea* found in sample		
	No. 1	No. 2	No. 3
pH 5.2 "wet"	10	28¼	9¼
pH 8.0 "wet"	½	10¼	1¼
"wet" blotter (tap water)	5¾	17½	6
"dry" blotter (tap water)	14¼	33	10¾

Other experiments in which the same buffer solutions were added in normal blotter tests gave similar results.

Addition of various compounds to the blotter

The observations mentioned also provided an explanation for the results of ANSELME *et al.* (1966). We communicated with these workers in 1964 on our findings with the WBE and the smaller difference between blotter tests and agar tests if the blotters were kept dry. These workers then tried the addition of 3% malt extract to the blotters and thus obtained results of the same level as that of the agar test. This can now be explained by the very low pH of malt extract. ANSELME *et al.* (l.c.) found it necessary to add 0.1% Brestan (20% triphenyltinacetate) in addition to the malt extract in order to check the growth of saprophytes. With our samples this was not necessary. However, also Brestan in itself has an effect on the WBE. This can be illustrated by the following average infections for five *Botrytis*-infected flax samples:

1. on "wet" blotters	8.9%
2. on "dry" blotters	16.8%
3. on "wet" blotters with 0.1% Brestan	15.9%
4. on "wet" blotters with 3% Difco Malt Extract	17.4%
5. on "wet" blotters with Difco Malt Extract Broth	17.7%
6. combination of 3 and 4	17.5%
7. combination of 3 and 5	18.1%

The apparent indifference to Brestan shown by *B. cinerea* did not apply to other fungal infections tested, viz. *Stemphylium radicinum* in carrot seed and *Alternaria*

brassicicola in cabbage seed. Moreover it was slightly toxic to the young flax seedlings. Also the addition of Duter (triphenyltinhydroxide) was tried, but this did not improve the results of "wet" blotter tests although it was apparently not very toxic to *B. cinerea* either. In MA Brestan up to concentrations of 1000 ppm could be added. Although this higher concentration stunted and retarded the growth of *B. cinerea*, it did not affect the ultimate percentages found to any great extent. KAARS SIJPESTEIJN *et al.* (1962) reported triphenyltinacetate to be especially toxic to Gram-positive bacteria and less to *Botrytis allii*.

Other infections than B. cinerea *in flax seed*

In the foregoing sections the conclusion was reached that the WBE for *B. cinerea* infection of flax seed could be explained on the basis of bacterial antagonism. The same was true for other infections as was shown by adding terramycin to the "wet" blotter tests for them also. This could be done up till now with the following seed infections: *Phoma exigua* var. *linicola* in flax seed, *Stemphylium radicinum* in carrot seed, and *Alternaria brassicicola* in cabbage seeds.

Infection	Number of samples tested	"Wet" blotter	"Dry" blotter	"Wet" blotter with 100 ppm terramycin
P. exigua var. *linicola* (flax)	7	5.7	10.3	15.7
S. radicinum (carrot)	12	19.7	24.9	25.6
A. brassicicola (cabbage)	12	50.2	62.5	59.1

As to the above infections of carrot and cabbage seed it should be remarked that these figures do not represent the entire picture. The expression of the pathogen was much weaker on the "wet" blotters than on the "dry" ones. The blotter test for *Stemphylium* in carrot seeds depends on the recognition of spores of that fungus. These were produced much more scarcely on seeds on "wet" blotters. With one carrot sample an experiment was carried out in which the effect of bacterial antagonism was further illustrated in the same way as had previously been done

TABLE 4. Results of experiments on the influence of bacterial antagonism in the blotter test for *Stemphylium radicinum* with one sample of carrot seed.

Resultaten van proeven over de invloed van bacterieel antagonisme in de filtreerpapierproef voor Stemphylium radicinum *met een monster wortelzaad.*

1. "Dry" blotters	46¾ %
2. "Wet" blotters	37¼ %
3. "Wet" blotters with sterile Difco Nutrient Broth	26½ %
4. As 3, but with rough bacterial culture from carrot seed added	20 %
5. "Wet" blotters with P-buffer of pH 5.2	60¼ %
6. "Wet" blotters with P-buffer of pH 8.0	35¾ %
7. "Wet" blotters with 100 ppm terramycin solution	63 %
8. As 3, but "dry"	49 %
9. As 5, but "dry"	70¼ %
10. As 6, but "dry"	47½ %
11. As 7, but "dry"	69¼ %

with flax samples as described in preceding sections. The treatments used and their results can be found in Table 4. In the above test the conditions 2, 3, 4 and 6 gave much development of *Trichothecium roseum* and no other fungi. In the conditions 5 and 7 and the "dry" treatments hardly any *T. roseum* was found, but the development of many other fungi was markedly increased *(Fusarium, Stachybotrys, Alternaria, Epicoccum)*.

Bacterial antagonism in seed infections without WBE

The *Fusarium* infection of wheat seed does not show a WBE, but it does show a soak-effect as has been indicated earlier. Further experiments carried out with wheat seed soaking showed that increasing the duration of soaking gave a further decrease of the percentage obtained. This could always be counteracted by means of terramycin, but with increased duration of the soaking period it proved to be necessary to increase the amount of terramycin to be added. It was interesting that the decrease of the infection percentage found with increased duration of soaking was more rapid for some samples than for others. Repetition of experiments always gave similar results for the same samples. This indicated that the reaction in the test was an inherent property of the sample. This is suspected to be due either to differences in kind and amount of antagonistic bac-

TABLE 5. Average percentages for the symptoms obtained with the blotter test for *Fusarium* spp. in wheat grains after various presoaking treatments.
A. Percentages for 26 samples soaked during 24 hours in water, in 100 ppm terramycin solution and non-presoaked control. B. Percentages for 5 samples soaked during 24 hours in water, 100 ppm terramycin solution, 200 ppm terramycin solution and non-presoaked. C. Percentages for 4 samples soaked during 24 hours in water with and without air being bubbled through, and of non-presoaked control.

Gemiddelde percentages verkregen met de filtreerpapierproef voor Fusarium *spp. in tarwekorrels na verschillende voorbehandelingen.*
A. Percentages voor 26 monsters gedurende 24 uur geweekt in water, in 100 ppm terramycine-oplossing en onge weekt. B. Percentages voor 5 monsters gedurende 24 uur geweekt in water, 100 ppm terramycine-oplossing en ongeweekt. C. Percentages voor 4 monsters geweekt gedurende 24 uur in water met en zonder doorleiden van lucht.

Pretreatment	Killed seeds	Severe symptoms	Light symptoms	Total *Fusarium*
A. Water-soak/*Geweekt in water*	2.01	5.05	16.39	23.45
100 ppm terramycin	3.89	10.78	19.79	34.64
Control/*Onbehandeld*	3.06	8.27	23.27	34.60
B. Water-soak/*Geweekt in water*	2.80	2.15	8.35	13.30
100 ppm terramycin	4.10	3.95	15.80	23.85
200 ppm terramycin	5.50	5.25	16.05	26.80
Control/*Onbehandeld*	2.65	3.25	17.00	22.90
C. Water-soak/*Geweekt in water*	0.25	2.25	15.00	17.50
Water-soak, aerated/*Geweekt in geaereerd water*	2.00	4.25	30.25	36.50
Control/*Onbehandeld*	1.75	3.00	32.25	37.00

Voorbehandeling	*Gedode zaden*	*Zware symptomen*	*Lichte symptomen*	*Totaal* Fusarium

teria being present or to a different sensitivity of the *Fusarium* infection. When air was bubbled through the soaking water by means of a small aquarium pump no decrease of infection could be noted, even after 48 hours. This points to the fact that anaerobiosis may play an important role in the "soak-effect", and perhaps also in the related WBE. The formation of a water film around smaller seeds on "wet" blotters may be thought to create such a condition. In Table 5C the results of soaking four samples of wheat seeds with and without air being bubbled through are given. Very interesting was the observation that soaking of wheat seeds in terramycin solution results in a shift from light symptoms to severe symptoms, as well as an increase in the number of "killed" seeds (that are non-germinating seeds on which *Fusarium* mycelium develops). This indicates that bacterial antagonism can also play a role in infections that do not show the WBE under normal testing conditions. The figures given in Table 5 A and B illustrate this.

DISCUSSION

The experiments taken indicate that the WBE in the blotter test is only important for those kinds of seeds that under the conditions of the health test can take up water rapidly, viz. mainly the small-seeded species. For larger kinds similar results can be obtained when water uptake is increased by special measures, such as soaking treatments, which are no current practice in seed testing (although occasionally practised in germination testing of certain seed species). According to experiments with the antibacterial antibiotic terramycin the reduced percentages of infection found for pathogenic fungi on "wet" blotters are the consequence of a stimulated development of antagonistic saprophytic seed-borne bacteria on the quickly swelling seeds.

The establishment of a water film around the seeds might favour anaerobiosis and thus stimulate the development of the normal bacterial seed flora. This antagonates the fungal flora including the fungal pathogens at which the health test is aiming. The anaerobic character of the bacteria concerned was suggested by experiments in which wheat seed was soaked in water through which air was led, after which they were subjected to a health test. There might be a connection between these observations and the method of controlling deep seated seed-borne infections by means of cold water treatments, suchs as developed since 1953 by TYNER and others in the United States and elsewhere.

SPICHER (1956) for wheat and EBNER (1960) for beet seed found that the development of the seed microflora during storage depends on the initial ratio between the bacterial and fungal groups. Bacterial development can be rather rapid, as was shown by VERONA (1963) in experiments in which seeds of wheat, flax, clover, alfalfa and cabbage were placed in a drop of liquid on a microscope slide during 24 hours. The main seed health testing methods are incubation methods (on blotters or agar, in sand, soil, etc.), in which conditions may be such that either the bacterial or the fungal seed flora is favoured. If one of the two dominates since early stages of the test, this will be reflected in the results. The ratio between the two can be influenced by moisture supply, temperature, pH, aeration and other factors. The differences in results between the blotter and agar methods for *Botrytis cinerea* in flax can readily be explained in this way.

It should be realized that the antagonistic microflora might be important for

the agricultural value of the seed, as it is sown together with the seed. In this connection may be referred to a publication by RANGASWAMI & RAMALINGAM (1962), who observed that *Helminthosporium oryzae* conidia survived for 131 days in sterile soil and 98 days in non-sterile soil, but when the conidia were used for artificial infection of seeds they survived for 37 days in sterile and 15 days in non-sterile soil only. Also, already before World War II, Russian workers tried with some success bacterization of flax seed for controlling flax diseases (NOVOGRUDSKII *et al.*, 1937; BERESOVA, 1939).

The influence of moisture on the development of the bacterial flora of wheat seeds was already demonstrated by SIMMONDS (1947). This author showed that incubation of wheat seeds on blotters in a moist chamber increased the degree of antibiosis against artificial infection by *Helminthosporium sativum*. This effect was not observed after formalin treatment of the incubated seeds before inoculation with *H. sativum*.

In view of the results reported in this paper the question arises whether in seed health testing antagonism should have its way or not. Should one take the "isolated view" of the "integrated view", in other words, try to determine the total pathogenic infection or the effective one? Or, returning to where the present investigation started, use the "wet blotter test" or the "dry blotter test" (or any other method of which the results correspond with either the former or the latter)?

The results obtained on "dry" blotters, or on "wet" blotters with antibiotics or with a similar method are more easy to standardize, but they might bear little relation to the significance of an infection for field performance of the seed. It is interesting in this respect that VAN DER SPEK (1965) reported a better correlation with disease development in non-sterile soil out of *Botrytis*-infected flax seed for the blotter method (not yet standardized as to substrate moisture!) than for the agar method.

Though for the time being it may be preferable to pursue maximum figures it may in the long run be better to produce figures of greater agricultural value. Experiments in soil will have to decide this issue. Of course, for plant quarantine purposes (viz., with regard to the introduction by means of infected seed lots of pathogens, that may develop into a serious threat to the importing country's economy) the most sensitive method will remain preferable. But for determining the sowing value of seed lots a more relative method producing lower figures may have great advantages. The farmer is interested in the potential threat that seed-borne infection is to his crop. A good example of an important pathogen causing an unimportant seed infection was recently given by SCHERMER & SCHIPPERS (1965) who found that *Verticillium albo-atrum* is seed-borne in *Senecio vulgaris*, but only seed-transmitted when the seed is incubated in sterile media.

It is long since known that the consequences of a certain seed-borne infection in field sowings are often very strongly influenced by sowing and growing conditions. It now appears that incubation tests for seed-health may also be strongly affected by testing conditions, and moreover, that the influence of outward conditions is often an indirect one and primarily caused by microbiological antagonism.

SAMENVATTING

DE TEMPE & LIMONARD (1966) vonden dat een hoog vochtgehalte van het filtreerpapier in de filtreerpapiermethode voor gezondheidsonderzoek van zaaizaad het voor bepaalde pathogene schimmels gevonden percentage aanzienlijk kan verlagen. Dit bleek bijv. het geval voor *Botrytis cinerea* in vlaszaad en *Alternaria brassicicola* in zaad van kool, maar niet voor *Phoma betae* in bietezaad en *Helminthosporium* spp. en *Fusarium* spp. in graanzaden. Om de oorzaak van de vochtinvloed op filtreerpapier („wet blotter effect" of „WBE") na te gaan werd het hier beschreven onderzoek ingesteld, waarbij vooral vlaszaad met *B. cinerea* werd gebruikt.

De resultaten van de aan zaad van vlas, kool, biet en tarwe na incubatie gedurende verschillende perioden op „nat" en „droog" filtreerpapier verrichte vochtbepalingen zijn vermeld in Tabel 1. Hieruit blijkt dat er een verschil in snelheid van wateropname bestaat voor verschillende zaadsoorten en voor eenzelfde zaadsoort op „nat" en „droog" filtreerpapier. De snelheid van wateropname en het verschil in vochtniveau op „nat" en „droog" filtreerpapier zijn het kleinste voor tarwe- en bietezaad. Infecties van deze zaadsoorten vertoonden het „wet blotter effect" niet. *B. cinerea* op tarwezaad bleek ook niet te reageren. Er werd daarom geprobeerd om de snelheid van wateropname bij deze zaadsoorten te vergroten door ze in water te weken. Weken van tarwezaad bij 20 °C verlaagde inderdaad de gevonden aantastingspercentages („soak-effect"). Weken van tarwe bij 10 °C had veel minder effect. Het water bleef helder en reukeloos bij deze temperatuur, doch niet bij 20 °C. Daarom werd vermoed dat bacteriële ontwikkeling de oorzaak was van de gevonden resultaten. Toevoeging van terramycine aan het weekwater gaf opheffing van het „soak-effect". Toevoeging van terramycine (of streptomycine) aan „nat" filtreerpapier bleek ook het „wet blotter effect" te doen verdwijnen. Voor vlaszaad met *B. cinerea* konden met de filtreerpapierproef op deze wijze dezelfde resultaten worden verkregen als met de agarproef (tabel 3). De filtreerpapierproef geeft normaliter lagere resultaten dan de agarproef. Blijkbaar treedt in de agarproef geen bacterieel antagonisme op. Gezien het verschil in pH tussen de gebruikte agar en het filtreerpapiermilieu werd nagegaan welke rol deze hierbij speelt. Hiertoe werd de pH van het filtreerpapiermilieu verlaagd of verhoogd door middel van bufferoplossingen. „Nat" filtreerpapier van pH 5,2 bleek een hoger percentage *Botrytis* te geven dan „nat" filtreerpapier met leidingwater (pH 6,9–7,0) en „nat" filtreerpapier van pH 8,0 een lager. Ook de resultaten verkregen door ANSELME *et al.* (1965) door toevoeging van moutextract aan het filtreerpapier kunnen nu worden verklaard als een pH-effect.

Op dezelfde wijze kon worden aangetoond dat het „wet blotter effect" voor *Phoma exigua* var. *linicola* in vlaszaad, *Alternaria brassicicola* in zaad van kool en *Stemphylium radicinum* in wortelzaad eveneens wordt veroorzaakt door bacterieel antagonisme.

In de discussie wordt gewezen op het feit dat een aantal factoren zoals snelle wateropname („wet blotter effect" en „soak effect") en hogere pH-waarden blijkbaar het evenwicht tussen bacteriën en schimmels in de zaadflora ten gunste van de eerste groep verschuiven. Er wordt gewezen op literatuur (SPICHER, 1956; EBNER, 1960) welke een negatieve correlatie tussen de ontwikkeling van deze beide groepen van micro-organismen in de zaadflora aangeeft. De vraag

wordt gesteld welke methode voor gezondheidsonderzoek van zaaizaden de voorkeur verdient, die waarbij bacterieel antagonisme wordt uitgeschakeld of onbelangrijk is (filtreerpapier met antibacteriële antibiotica of agar) of de methode waarbij dit wordt gestimuleerd („nat" filtreerpapier). Een en ander kan niet los worden gezien van de vraag of men een gemakkelijk reproduceerbaar maar landbouwkundig gezien minder waardevol cijfer wenst, dan wel een minder gemakkelijk reproduceerbaar maar voor de uitzaaiwaarde-beoordeling belangrijker resultaat.

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