NN 8201 no 437 C

ECOLOGICAL ASPECTS OF SEED HEALTH TESTING

MONARD

BIBLIOTHEEK
DER
LANDBOUWHOGESCHOOL
WAGENINGEN.

NN08201,437

ECOLOGICAL ASPECTS OF SEED HEALTH TESTING

Dit proefschrift met stellingen van

THEUNIS LIMONARD,

landbouwkundig ingenieur, geboren te Hoogland, 17 november 1933, is goedgekeurd door de promotor, Dr. A. J. P. Oort, hoogleraar in de plantenziektenkunde.

De Rector Magnificus van de Landbouwhogeschool,
F. HELLINGA

Wageningen, 24 oktober 1968

631.53.011.5:632.4.093.2

ECOLOGICAL ASPECTS OF SEED HEALTH TESTING

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN
AAN DE LANDBOUWHOGESCHOOL
TE WAGENINGEN OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. IR. F. HELLINGA, HOOGLERAAR IN DE CULTUURTECHNIEK,
IN HET OPENBAAR TE VERDEDIGEN IN DE AULA
VAN DE HOGESCHOOL OP VRIJDAG 13 DECEMBER 1968
TE 16 UUR

DOOR

T. LIMONARD

1968
INTERNATIONAL SEED TESTING ASSOCIATION, WAGENINGEN, NETHERLANDS

PROMOTOR: Prof. Dr. A. J. P. Oort

The present investigation was carried out at the Rijksproefstation voor Zaadcontrôle (Official Seed Testing Station), Wageningen, under the supervision of Dr. J. de Tempe, Head of the Department of Seed Health Testing
This thesis is also published in the Proceedings of the International Seed Testing Association, Vol. 33, No. 3.

Aan mijn vrouw

STELLINGEN

I

Chemische zaadontsmetting dient uitsluitend te worden toegepast indien dat strikt noodzakelijk is.

II

De mening van L. B. JOHNSON *et al.* dat de resultaten van hun onderzoekingen pleiten tegen de aanwezigheid van „mobilisatiefactoren" in sporen van *Puccinia recondita* is aan ernstige bedenkingen onderhevig.

L. B. JOHNSON *et al.*, Phytopathology 56: 799 (1966)

III

Er wordt onvoldoende rekening gehouden met het feit dat het gebruik van herbiciden het optreden van planteziekten kan bevorderen.

IV

Inoculatieproeven met aaltjes onder uitsluiting van andere fytopathogenen zijn ongeschikt om over hun schadelijkheid t.o.v. planten te beslissen.

V

Daar de belangrijkste eigenschappen van plantevirussen door hun nucleïnezuurkern worden bepaald is het nut van serologische reacties voor het onderscheiden van virusstammen beperkt.

C. I. KADO & C. A. KNIGHT, Proc. Nat. Acad. Sci. 55: 1276 (1966)

VI

De recente ontdekking dat aster-yellows waarschijnlijk niet door een virus maar door een mycoplasma wordt veroorzaakt, maakt het verschijnsel van waardreeksvergroting van *Dalbulus maidis* („corn leaf hopper") na infectie met de veroorzaker van aster-yellows begrijpelijker.

Y. DOI *et al.*, Ann. Phytopath. Soc. Japan 33: 259 (1967)
K. MARAMOROSCH, Tijdschr. Plziekt. 64: 383 (1958)
S. W. ORENSKI & K. MARAMOROSCH, Phytopathology 52: 1219 (1962)

T. LIMONARD
Wageningen, 13 december 1968

VII

De isolering van *Streptomyces alni* door FIUCZEK en DANILEWICZ en de met dit organisme beschreven proeven zijn zeer aanvechtbaar.

M. FIUCZEK, Acta Microbiol. Polon. 8: 283 (1959)
K. DANILEWICZ, Acta Microbiol. Polon. 14: 321 (1965)

VIII

Dat haemoglobine betrokken zou zijn bij het mechanisme van de symbiontische stikstofbinding door *Rhizobium* spp. is nog zeer de vraag.

F. J. BERGERSON & G. L. TURNER, Biochim. biophys. Acta 141: 507 (1967)

IX

„Micro-encapsulation" van insecticiden heeft voordelen maar kan het residuprobleem vergroten.

F. T. PHILLIPS, PANS 14: 407 (1968)

X

De huidige inrichting van de studie (t/m de promotie) aan de LH voldoet uit een oogpunt van efficiëntie en duidelijkheid minder dan die aan overeenkomstige instellingen in Noord-Amerika.

XI

Al het onderwijs te beginnen bij de lagere school zou dienen te worden gecentreerd rondom een studie der wijsbegeerte, met name een bezinning op het wezen van de mens.

VOORWOORD

Bij het tot stand komen van dit proefschrift wil ik gaarne allen danken die hieraan hun medewerking hebben verleend.

Zeer bijzondere dank en erkentelijkheid ben ik verschuldigd aan dr. J. de Tempe, hoofd van de afdeling Gezondheidsonderzoek van het Rijksproefstation voor Zaadcontrôle te Wageningen, alwaar dit onderzoek werd verricht. Hij was het die mij inwijdde in de zaadpathologie en haar problemen en mij altijd zeer bereidwillig met raad en daad terzijde stond.

Grote dank en erkentelijkheid ben ik vooral ook verschuldigd aan mijn promotor, prof. dr. A. J. P. Oort, op wiens voorstel ik met dit onderzoek begon. Ik ben hem zeer dankbaar voor de wijze waarop hij mij indertijd hielp bij het voltooien van mijn studie in Wageningen en voor de gelegenheid die hij mij geboden heeft om na het beëindigen van dit onderzoek onder zijn leiding te komen werken.

Dr. A. F. Schoorel, directeur van het Rijksproefstation voor Zaadcontrôle ben ik zeer dankbaar voor de verleende gastvrijheid en voor zijn stimulerende interesse en grote hulpvaardigheid.

Mevr. J. Brouwer-Jansen, mevr. I. Kliffen-Wassenaar en mej. M. Peplinska dank ik voor de ijver en toewijding waarmede zij bij het onderzoek hebben geassisteerd.

Het gehele verdere personeel van het R.P.v.Z., in het bijzonder dat van de afd. gezondheidsonderzoek, de kas, de werkplaatsen en de schoning, ben ik dank verschuldigd voor de wijze waarop zij steeds bereid waren mij te helpen.

De verschillende gewestelijke laboratoria van de N.A.K. en buitenlandse collegae ben ik zeer dankbaar voor hun hulp bij het verkrijgen van interessante zaadmonsters. De afd. Wiskunde van de Landbouwhogeschool, in het bijzonder de heer M. Keuls, dank ik voor hun adviezen bij het verwerken van een gedeelte van het cijfermateriaal.

Ir. J. van der Toorn van het Laboratorium voor Microbiologie van de Technische Hogeschool te Delft ben ik erkentelijk voor zijn identificatie van *Bacillus cereus* var. *mycoides.*

Ten slotte wil ik mijn oprechte dank betuigen aan het Amerikaanse Ministerie van Landbouw, dat dit onderzoek financieel mogelijk maakte.

CONTENTS

1 INTRODUCTION 1

2 MATERIALS, METHODS AND EQUIPMENT 4
2.1 Materials 4
2.2 Methods 5
2.2.1 Blotter method 10
2.2.2 Agar method 11
2.2.3 Sand and soil tests 12
2.3 Equipment 12
2.3.1 Incubators 12
2.3.2 Transfer room and sterilizing equipment 13
2.3.3 Greenhouse and climate cells 13
2.4 Use of scientific names 14

3 BACTERIAL ANTAGONISM 14
3.1 Introduction, the 'wet blotter effect' (WBE) 14
3.2 Prevalence and importance of the WBE 14
3.3 Is the WBE a fungistatic or a fungicidal phenomenon? 16
3.4 The relative importance of pathogen and seed in the WBE 17
3.5 The water uptake of different kinds of seeds on 'wet' and 'dry' blotters 18
3.6 The 'soak effect' (SE) for seeds without WBE 21
3.7 The influence of temperature on the WBE 22
3.8 The influence of antibacterial antibiotics on the WBE 23
3.9 Experiments with stimulation of bacteria 23
3.10 Experiments on the influence of pH 24
3.11 Blotter experiments with malt extract and Brestan 25
3.12 Examination of the difference between blotter and agar test results for *Botrytis cinerea* in flax seed 26
3.13 Experiments on the WBE for some other infections 29
3.14 The influence of terramycin on the blotter test results for *Fusarium* spp. in wheat seed 29
3.15 Additional experiments on the SE with wheat seed and the isolation of one of the implicated bacterial species 30
3.16 Mass and individual soak experiments and the occurrence of positive soak effects 33

3.17 The influence of aeration on the SE 36
3.18 The influence of soaking on agar test results 38
3.19 The influence of hypochlorite pretreatment on the WBE and SE . . . 42
3.20 Bacterial antagonism in the agar method 43
3.21 The moisture effect in silversand and soil 46
3.22 The effect of soaking, bacterization and other pretreatments of seeds on disease development in silversand and soil 48
3.23 Discussion 50

4 HOST VIGOUR 54
4.1 Introduction 54
4.2 The use of 2,4-D in seed health testing 54
4.3 The 2,4-D effect 55
4.4 Influence of 2,4-D on fungi 56
4.5 The mechanism involved in the 2,4-D effect for the *Phoma valerianellae* infection of corn-salad seed 58
4.6 The factor 'host vigour' for *Phoma valerianellae* infection of corn-salad seed in soil 61
4.7 Host vigour and *Phoma betae* infection of beet seed 62
4.8 Host vigour and *Phoma lingam* infection of cabbage seed 64
4.9 Host vigour and infections carried by wheat seed 65
4.9.1 2,4-D experiments 65
4.9.2 Experiments with the freezing method 66
4.9.2.1 *Fusarium* spp. 66
4.9.2.2 *Septoria nodorum* 69
4.9.2.3 *Helminthosporium* spp. 71
4.10 Host vigour and infections of oat and barley seeds 71
4.11 Host vigour and infections of other kinds of seed 73
4.12 The perspex-freezing method 75
4.13 Discussion 76

5 INTERFUNGAL ANTAGONISM 78
5.1 Introduction 78
5.2 The effect of temperature 78
5.3 The use and effect of superficial disinfection with hypochlorite 84
5.4 The effect of the removal of external 'seed' structures 88
5.5 The dry heat treatment 91
5.6 Experiments with selective compounds 94

5.7 Experiments with growth retardants 101
5.8 Some experiments with artificial inoculation 103
5.9 Discussion 106

6 Inoculum potential 107
6.1 Introduction 107
6.2 NaClO pretreatment and inoculum potential 109
6.3 The use of the Christensen method 111
6.4 Importance of glume infections 113
6.5 Discussion 114

7 The use of correlation coefficients 116
7.1 Introduction 116
7.2 Methods used and the figures obtained 117
7.3 Conclusions drawn from results of previous section 123
7.4 Discussion 129

8 Discussion 133
8.1 Factors affecting the seed microflora 133
8.2 Practical consequences for the choice of seed health testing methods . 141

Acknowledgements 146

Summary 146

Samenvatting 149

Résumé 152

Zusammenfassung 156

References 160

1 INTRODUCTION

Many important plant pathogens can be spread with the seed of their host plants through time and space. The annotated list of seed-borne diseases by NOBEL *et al.* (1959), of which a revised edition is in press, gives an idea of the occurrence of seed transmission among plant pathogens. It is quite evident, therefore, that the detection of seed-borne infections is very important.

Notwithstanding its importance, testing for seed health has been much neglected in comparison with that for other seed qualities such as germinability and purity. NEERGAARD (1965) has given some possible reasons for this neglect. One of the main reasons has been the lack of sufficiently reliable methods. A very important feature of seed testing in general is the aiming at exact reproducibility. The methods which are up till now available for seed health testing, leave much to be desired in this respect, apart from the fact that for many seed-borne diseases no good routine methods are known as yet.

The Plant Disease Committee (P.D.C.) of the International Seed Testing Association (ISTA) has been quite active over the last fifteen years in trying to ameliorate this situation. The above committee consists of seed pathologists from a number of countries, which are carrying out routine seed health testing in their home laboratories. They use a range of methods, conditions and criteria.

One of the tasks that confronts the P.D.C., therefore, is the standardization of conditions and criteria and the decision about agreed methods. One of the ways in which it goes about this task is by international comparative testing of infected seed samples, the results of which are compared and discussed by the P.D.C.-members at their yearly workshops. Both the referee tests and workshops are organized by its very active chairman, Dr. P. NEERGAARD of Denmark. Although the above activities have undoubtedly resulted in an improvement of the existing situation, they are not a sufficient remedy because of the lack of underlying research work. Merely comparing existing methods and then deciding on arbitrary grounds about the best one for official international recommendation, is not a very satisfactory procedure. For the time being, however, it was the best that could be done.

However, the results of the 'referee tests' for most infections showed a deplorable lack of agreement (P.D.C. – ISTA reports). To quote BAKER & SMITH (1966), the methods used in the above comparative tests 'provide satisfactory evidence for seed transport, but the variability of the results indicates that the quantitative information on the incidence of a given pathogen may be of limited

reliability'. It should be added that the P.D.C. activities included only a small number of tests each year, the results of which were obtained by a painstaking and time-consuming study. In actual routine testing much less time can be spent on each sample.

The deplorable situation described above can only be expected to be improved by research. It was on the instigation of Dr. O. L. JUSTICE, previous ISTA president and many years director of the Federal U.S.A. Seed Testing Station at Beltsville (Md.), that the U.S. Department of Agriculture (U.S.D.A.) granted the Netherlands Government Seed Testing Station at Wageningen the necessary funds to carry out research on this subject, to be paid for under the terms of the U.S.A. Public Law 480.

The choice of Wageningen was no surprise. At this station the routine examination of commercial samples was first started, as early as 1918, by Dr. L. C. DOYER, who established a separate Seed Health Testing Department. At this department, which has been directed since 1949 by Dr. J. DE TEMPE, momentarily 3000 to 4000 commercial samples of mainly agricultural seeds are tested each year.

The author was asked to join the above department for research on the project under the terms of the agreement with the U.S.D.A. His first problem was to become acquainted with the existing methods and problems. The former could be divided into two categories, as done already by DOYER (1938):

A. Direct methods, in which the structures of the pathogen or symptoms of the disease can be observed directly on the dry or soaked seeds, in shakings, etc., either macroscopically or microscopically. These methods have the advantage of being fast, but they fail to indicate many extremely important mycelial infections; moreover no distinction can be made between living and dead infection. Hence those methods are, with a few exceptions (e.g. the embryo method for loose smut of barley and wheat, shaking methods for bunt and covered smuts of cereals, the water drop method for blind seed disease of ryegrasses), of limited value.

B. Incubation methods, in which the seeds are incubated for a certain period on, or in, a certain medium under specific environmental conditions, in order to allow the pathogen to express its presence. This latter can be either by the appearance of symptoms of the disease, or of signs of the pathogen itself. An important condition is that these symptoms or signs should be sufficiently characteristic for an easy and correct recognition.

It was decided to restrict our investigations to this second group and to fungal pathogens. It is this group with which the ISTA – P.D.C. has up till now mainly concerned itself and in which the results were so unsatisfactory. The fact that the participants, while agreeing at the workshops with each other about the characters to be looked for, did arrive at different results at home – notwithstanding standardized conditions of duration, temperature, light, etc., – called for an explanation.

The incubation method exclusively used at Wageningen for routine work at the beginning of the present investigations was the blotter method, described in chapter 2.

In most other laboratories, with only a few exceptions, the blotter method was also the most important one (NEERGAARD, 1965). It was the blotter method, therefore, which received the author's first attention. The so-called 'wet blotter effect', described in chapter 3, served as a starting point. The study of this and other problems led to the view that the factors influencing the results of incubation tests were far more complex than was originally expected. This study showed some reasons why the results of incubation tests can be so variable.

It was realized more and more that seeds can harbour quite a lot of different organisms at the same time, which may enter into various relationships among themselves and with the germinating seed. This being so, the seed can be regarded as a complex ecological unit, a 'biotope'. As long as the seed is stored in a proper manner most of the members of the seed microflora as well as the seed itself remain in a dormant condition. When the seed is incubated, however, a large part of them will awake and start growing, and the above interactions will start. The incubation conditions provided will not only directly influence the pathogen we want to detect, but also indirectly via their effect on the interactions. Chapters 3, 4 and 5 will deal with some components of the biotope and their significance in incubation tests. In those chapters the relative importance of these components in various kinds of incubation tests will be compared. This study also indicated ways to avoid these difficulties, and possibly leading to new or modified methods.

In seed health tests the results are ordinarily expressed as percentages of infected seed. An exception is, e.g., the shaking method for bunt and covered smut in cereals, where the results are expressed by the number of spores obtained from a certain number of seeds. In chapter 6 attention will be given to the load of the pathogen present on each seed, as this 'inoculum potential' may be expected to be at least as important for disease development in the field, as the percentage of infected seeds.

The agricultural value of the test results is another matter requiring investigation. The relation between field outbreaks of a disease and its seed-borne origin is often difficult to prove. At Wageningen one has always been particularly concerned about the significance of seed health testing results in terms of sowing value. Here the greatest value was attached to methods giving the highest correlation with disease development (DE TEMPE, 1963b, 1963). It was hoped, therefore, that the present work would lead to better correlations with field performance. Because of the complications revealed (chapter 7) and the fact that it is useless to correlate the results of unreliable laboratory tests with field performance, the comparison of various methods for the detection of certain seed-borne infections with disease development in the field had to be postponed.

Finally, in chapter 8, a general discussion on some aspects of the findings will be presented.

Seed health testing is, apart from its use for judging the agricultural value of the seed, also important for quarantine purposes. This latter aspect was not directly at stake in the present investigations. Quarantine methods need to be especially sensitive. Much of the present work was concerned with sensitivity of methods. It can thereby safely be assumed that if one method demonstrates the presence of e.g. 30% infection and another one that of 50% in the same sample, the second one is more suited for quarantine purpose, especially in case of traces of infection.

2. MATERIALS, METHODS AND EQUIPMENT

2.1 MATERIALS

The investigations described were carried out in the department of seed health testing of the Netherlands Government Seed Testing Station. Necessarily the investigations remained largely restricted to seed-borne infections that were prevalent year after year in the Netherlands. In the first place suitable samples could be found by relying on the department's routine inspection of commercial seeds. As a result infections of agricultural seeds were emphasized. When a sample had been proved to be interesting it was sometimes possible to obtain a sufficient amount of the corresponding seed lot. Large series of wheat seed samples containing *Fusarium* spp. were obtained by the kind cooperation of the

regional laboratories of the General Netherlands Inspection Service for Agricultural Seeds (N.A.K.). This made it possible to pay special attention to that important infection. Occasionally interesting samples were obtained from abroad. Sometimes special surveys were carried out using commercial samples for which no health investigation had been requested, and in this way interesting material was collected. The samples used always consisted of cleaned seed.

The aim of the author was to study methods and for this purpose it was not always possible to use economically important infections. Occasionally interesting observations were made with less important ones (table 1).

A difficulty was the gradual change of samples during storage. The results obtained with the same samples at different times cannot always be directly compared. So in each experiment suitable controls had to be included.

The term 'seed', as used in this publication, refers to sexually derived structures of spermatophytes which can germinate to produce new plants, unless otherwise indicated. This corresponds to its normal use in the world seed industry.

2.2 Methods

The present work was restricted to a study of the incubation methods for seed-borne fungi (cf. ch. 1). These methods can be sub-divided according to the medium on or in which the incubation takes place. Thus we can speak of blotter, agar, sand or soil tests, of which the first two are used most frequently. When this work was started the only incubation method used for routine health testing at Wageningen was the blotter method.

Each method has various sources of error. In the first place there is the error due to sampling and sub sampling of the sample, usually 400 seeds being used per object. Secondly there is the error caused by false judgment, due to the subjective element (each method has its borderline cases of doubtful symptoms or signs). Finally there is the error characteristic for the incubation methods, namely that evolving from insufficient knowledge and control of developments during incubation. The latter error happens to be inherent to this group of methods having the broadest applicability and thus being most important for seed health testing.

The present investigation dealt with that particular source of error by mainly studying the ecology of incubation methods.

The results of seed health tests are usually expressed as percentages of seeds found to harbour a particular pathogen. In this paper a comparison of methods

TABLE 1. The routine blotter test conditions applied at the start of the present work by the Wageningen Seed Testing Station for those infections that were studied (some frequently, others only occasionally). Frequently used synonyms and perfect states of the pathogens are also indicated.

Seed	Pathogen	Test-duration and temperature	Light conditions	Observations
Allium spp	*Botrytis* spp.	10 d. 20°C	NUV	mycelium + spores
	Stemphylium botryosum WALLR. perf. state *Pleospora herbarum* (PERS. EX. FR.) RABENH.	10 d. 20°C	NUV	spores
Avena sativa L.	*Fusarium* spp.	3 d. 10° + 3 d. 20°C	darkness	symptoms, see text
	Drechslera avenae (EIDAM) SCHARIF syn. *Helminthosporium avenae* EIDAM perf. state *Pyrenophora avenae* ITO & KURIBAY.	10 d. 20°C	NUV	spores
	Helminthosporium sativum P., K. & B. perf. state *Cochliobolus sativus* (ITO & KURIBAY.) DRECHS. EX DASTUR	10 d. 20°C	NUV	spores
Beta vulgaris L.	*Colletotrichum* sp.	12 d. 20–30°C	darkness	acervuli
	Fusarium spp.	12 d. 20–30°C	darkness	mycelium + spore masses
	Phoma betae FRANK perf. state *Pleospora betae* BJÖRLING	12 d. 20–30°C	darkness	pycnidia
Brassica spp.	*Alternaria brassica* (BERK.) SACC.	10 d. 20°C	NUV	spores
	Alternaria brassicicola (SCHW.) WILTS.	10 d. 20°C	NUV	spores
	Botrytis cinerea PERS. EX FR. perf. state *Sclerotinia fuckeliana* FUCKEL	10 d. 20°C	NUV	mycelium + spores
	Fusarium spp.	10 d. 20°C	NUV	mycelium + spore masses

	Plenodomus lingam (TODE EX FR.) HÖHN. syn. *Phoma lingam* (TODE EX FR.) DESM. perf. state *Leptosphaeria maculans* CES. & DE NOT.	10 d. 20°C (+2,4D)	NUV	pycnidia
Daucus carota L.	*Alternaria dauci* (Kühn) GR. & SK.	10 d. 20°C	NUV	spores
	Fusarium sp.	10 d. 20°C	NUV	mycelium + spore masses
	Stemphylium radicinum (M., & E.) NEERG.	10 d. 20°C	NUV	spores
Festuca pratensis HUDS.	*Helminthosporium* spp.	10 d. 20°C	NUV	spores
Hordeum vulgare L.	*Fusarium* spp.	3 d. 10°C+ 3 d. 20°C	darkness	symptoms, see text
	Drechslera graminea (RAB. EX SCHLECT.) SHOEMAKER syn. *Helminthosporium gramineum* RABENH. perf. state *Pyrenophora gramineum* RABENH.	10 d. 20°C	NUV	spores
	Drechslera siccans (DRECHSL.) SHOEMAKER syn. *Helminthosporium siccans* DRECHSL.	10 d. 20°C	NUV	spores
	Drechslera teres (SACC.) SHOEMAKER syn. *Helminthosporium teres* SACC. perf. state *Pyrenophora teres* DRECHSL.	10 d. 20°C	NUV	spores
	Helminthosporium sativum P., K. & B. perf. state *Cochliobolus sativus* (ITO & KURIBAY.) DRECHS. EX DASTUR	10 d. 20°C	NUV	spores
Lactuca sativa L.	*Botrytis cinerea* PERS. EX FR. perf. state *Sclerotinia fuckeliana* (DE BARY) FUCKEL	10 d. 20°C	Light NUV	mycelium + spores
Linum usitatissimum L.	*Alternaria linicola* GR. & SK.	10 d. 20°C	NUV	spores
	Botrytis cinerea PERS. EX FR. perf. state *Sclerotinia fuckeliana* FUCKEL	10 d 20°C	Light NUV	mycelium + spores
	Fusarium spp.	10 d. 20°C	NUV	mycelium + spore masses

Table 1 (continued)

Seed	Pathogen	Test-duration and temperature	Light conditions	Observations
	Phoma exigua Desm. var. *linicola* (NAOUM. & VASS.) MAAS syn. *Ascochyta linicola* NAOUM. & VASS.	10 d 20°C	NUV	pycnidia
Lolium spp.	*Helminthosporium* spp.	10 d. 20°C	NUV	spores
Lycopersicon esculentum MILL.	*Diplodina lycopersici* KLEB. perf. state *Didymella Lycopersici* HOLLOS	14 d. 15°C	NUV	pycnidia
Medicago & *Melilotus* spp.	*Ascochyta* spp. & *Phoma* spp.	10 d. 20°C	NUV	pycnidia
	Botrytis cinerea PERS EX FR. perf. state *Sclerotinia fuckeliana* FUCKEL	10 d. 20°C	NUV	mycelium + spores
	Colletotrichum trifolii BAIN & ESS.	10 d. 20°C	NUV	acervuli
	Stemphylium spp.	10 d. 20°C	NUV	spores
Papaver spp.	*Dendryphion penicillatum* (CDA) FR. syn. *Helminthosporium papaveris* SAW. perf. state *Pleospora papaveracea* (DE NOT.) SACC.	14 d. 20°C	NUV	spores
Pisum spp.	*Ascochyta pinodes* JONES perf. state *Mycosphaerella pinodes* (BERK. & BLOX.) VESTERGR.	5–7 d. 20°C	± light	symptoms, see text
	Ascochyta pisi Lib.	5–7 d. 20°C	± light	symptoms, see text
	Botrytis cinerea PERS. EX FR. perf. state *Sclerotinia fuckeliana* FUCKEL	5–7 d. 20°C	± light	mycelium + spores
	Fusarium spp.	5–7 d. 20°C	± light	mycelium + spore masses
	Stemphylium botryosum WALLR. perf. state	5–7 d. 20°C	± light	symptoms, see text

	Pleospora herbarum (PERS. EX FR.) RABENH.			
Raphanus sativus L.	*Alternaria raphani* GR. & SK.	10 d. 20°C	NUV	spores
Secale cereale L.	*Fusarium* spp.	3 d. 10° + 3 d. 20°C	darkness	symptoms, see text
Spinacia oleracea L.	*Botrytis cinerea* PERS. EX FR.	10 d. 20°C	NUV	mycelium + spores
	perf. state			
	Sclerotinia fuckeliana FUCKEL			
	Colletotrichum spinaciae ELL. & HALST.	10 d. 20°C	NUV	acervuli
	Phoma betae FRANK	10 d. 20°C	NUV	pycnidia
	perf. state			
	Pleospora betae BJÖRLING			
Trifolium spp.	*Ascochyta* spp. & *Phoma* spp.	10 d. 20°C	NUV	pycnidia
	Botrytis cinerea PERS. EX FR.	10 d. 20°C	NUV	mycelium + spores
	perf. state			
	Sclerotinia fuckeliana FUCKEL			
	Fusarium spp.	10 d. 20°C	NUV	mycelium + spore masses
	Stemphylium spp.	10 d. 20°C	NUV	spores
Triticum aestivum L.	*Fusarium* spp.	3 d. 10° + 3 d. 20°C	darkness	symptoms, see text
	Helminthosporium sativum P., K. & B.	10 d. 20°C	NUV	spores
	perf. state			
	Cochliobolus sativus (ITO & KURIBAY.) DRECHS. EX DASTUR			
	Helminthosporium spp.	10 d. 20°C	NUV	spores
	Septoria nodorum BERK.	14 d. 10°C (rather dry)	darkness	symptoms, see text
	perf. state			
	Leptosphaeria nodorum MÜLLER			
Valerianella locusta L.	*Botrytis cinerea* PERS. EX FR.	10 d. 20°C	NUV	mycelium + spores
	perf. state			
	Sclerotinia fuckeliana FUCKEL			
	Phoma valerianellae GINDRAT *et al.*	10 d. 20°C	NUV	pycnidia
Zea mays L.	*Fusarium* spp.	5 d. 25°C	darkness	mycelium + spore masses

and their modifications is mainly based on the difference in percentage obtained. The outcome of experiments is frequently presented in terms of average percentages. It can then be considered as the result of a larger sample consisting of a mixture of all the tests carried out. This was only done, however, when there were no samples showing different trends. In the latter case no positive conclusion was drawn, but the reason for the different reactions was investigated. The experiments were mostly repeated many times with the same and/or other samples. Similar results were always obtained provided no other factors interfered. They were also frequently sustained or confirmed by the results of related experiments.

2.2.1 *Blotter method*

The blotter method as performed at Wageningen is carried out on white blotter papers of about 10 × 26 cm underneath which a thick grey blotter (coarse paper wadding) is placed to serve as moisture stabilizer. These blotters are wetted by dipping them in tap water and are then put into perforated zinc or stainless steel trays. On the blotters ordinarily 100 seeds were placed, but it was soon evident that for most infections this number had to be reduced to 50 or less in order to avoid errors due to secundary spread of the pathogen.

The seeds are incubated, depending on the infection, for a specific number of days at a specific temperature, either in the dark or under near-ultraviolet (NUV) light, in special incubators as described in section 2.3.1. In table 1 the data about test duration, temperature and light conditions used in the routine tests of the Wageningen Station at the time when this work was started can be found. The routine methods were used as points of reference with which the results of other methods were compared.

The purpose of the incubation in blotter and other methods is the appearance of symptoms or signs by which the disease or the pathogen can be identified. Inspection after incubation is sometimes possible by naked eye (e.g. *Botrytis cinerea* in flax seeds), but usually a stereoscopic microscope of 20 to 30× magnification is required. In case of most of the infections of table 1, signs of the pathogens (fructifications, spores and sometimes characteristic mycelium) are looked for. Three exceptions are mentioned below.

In case of *Fusarium* spp. in cereal seeds the seedlings are inspected for the symptoms of brown discoloration of the roots, starting from the seed, as well as for the presence of characteristic mycelium and sometimes spore masses on non-germinated seeds. The symptoms are divided into two groups, viz. light ones, in which the root morphology is not seriously affected, and severe ones,

in which the rootlets have become stunted. The latter group would be classified as abnormal in germination tests (cf. DE TEMPE, 1964).

In the blotter test for *Septoria nodorum* in wheat, the seedlings are inspected for the presence of typical warts (protuberances) and lesions on the coleoptyles as well as distortions of the latter (cf. KIETREIBER, 1962, 1966).

Blotter tests for pea seed infections are inspected for the presence of characteristic brown spots caused by *Ascochyta* spp., and for yellow to reddish ones caused by *Stemphylium botryosum*.

These spots can be more eaily identified by cutting the seeds across the spots in two.

2.2.2 *Agar method*

In the agar methods seeds are placed on nutrient agar (usually potato dextrose agar, PDA, or malt extract agar, MA) in petri dishes. Before the seeds are plated out, it is often necessary to treat them with a mild disinfectant – usually hypochlorite solutions – in order to prevent rapidly growing surface-borne saprophytic fungi from obscuring the pathogens. At the Wageningen Station usually a pretreatment for 10 minutes with a ½ % NaClO solution is used. The seeds are then incubated under conditons dependent on the infection tested. As in these tests the purpose of the conditioned incubation is to induce the pathogen to betray its presence by recognizable development on the agar, growth should be in some way characteristic enough for identifying the pathogens. Mycelial growth does not need light and may even be inhibited by it but is often insufficient for identification. Therefore NUV-light is applied during the last 2 or 3 days of incubation. It serves to stimulate fructification of the colonies, which is quite important for a correct identification. Normally plastic petri dishes were used. One of their advantages is that they are NUV-transparant. Sometimes naked-eye inspection of the dishes is satisfactory, but often stereoscopic inspection at a 20 to 30 × magnification in transmittent light is necessary.

When the present investigations were started the blotter method was nearly exclusively used in certain seed testing laboratories, and the agar method in others; also there were stations that used either one or the other depending on the infections tested for. The reasons of preference are partly of a historical and partly of a practical nature, viz. equipment and costs (LIMONARD, 1967b). During the course of this work ways and means were found reducing the importance of the economical and technical restrictions concerning the agar method. Historical reasons have only a sentimental value in this connection and thus should not be decisive. The devices ultimately used for simplifying the agar

method have already been published (LIMONARD, 1967b). They mainly amount to application of a simple method of sterilization by means of propylene oxide, the large scale use of plastic dishes, and the use of a transfer room provided with an electrostatic air cleaner.

2.2.3 *Sand and soil tests*

Under sand tests we will understand those in so-called silversand; soil tests are those in true soils. Both sand and soil tests were often carried out in aluminium boxes of 14 × 17 cm surface and a depth of 3½ cm. Incubation in sand or soil is not ideal, as it prevents fungus observation so that one has to rely on symptoms caused on the seedling whereas part of the seeds may not produce an emerging seedling. The only ways by which the pathogen usually manifests its presence in these tests is reduction of the percentage of emergence and/or disease symptoms on the seedlings. In such tests these percentages were noted. One of the difficulties is a correct interpretation of disease symptoms that frequently are not very characteristic. Sometimes more precise observations were made by further incubating pieces of diseased tissue on moist filterpaper under near-ultraviolet light, or on agar plates.

The tests were also sometimes performed in plastic boxes of 32 × 32 cm size with a depth of 12 cm, in the climate cells mentioned in section 2.3.3.

For sand or soil tests under more or less sterile conditions the aluminium trays were provided with a wire framework of sufficient height, and sterilized in an electric oven. The trays were then filled inside the transfer room (cf. 2.3.2) with the medium, provided with the seeds and enveloped in a plastic bag which was sealed. Wet paper wadding underneath the trays served for maintenance of a high relative air humidity These units were indicated as 'tents' (Plate I, Fig. 1). They were incubated under the desired conditions, free from loss of water and protected from air-borne contaminations, so that quite simple incubators were sufficient In fact these tents were often placed under NUV tubes on open shelves in the laboratory room at the fairly constant room temperature of 20°C.

2.3 EQUIPMENT

The equipment used was that of the Seed Health Testing Department. We will briefly describe the main items below.

2.3.1 *Incubators*

The incubators used had been designed and built by the Station and consisted of two compartments into each of which 10 shelves could be placed at a mutual

distance of 12 cm. On each of these shelves 8 of the trays, used for the blotter test, could be placed. Both sides of the compartments had a double layer of quartz panes, between which either cooled or heated air was forced for temperature regulation. Behind the quartz panes two fluorescent tubes were mounted in a vertical position. These were either Philips fluorescent day light – Ph TL 40W 55 –, or General Electric F 40 BL – tubes. The latter were used in case near-ultraviolet (NUV) radiation was required. This NUV light of around 300 mμ induces sporulation in many species of fungi (LEACH *et al.*, 1959, 1962, 1967a, 1967b). Light was always given in a cycle of 12 hours darkness and 12 hours light. The bottom of the compartments was filled with a layer of water covered by a glass plate. A very slight air current was forced through a pipe in the centre of the bottom against the underside of the glass. This created an air movement over the water surface escaping at the edges of the glass plate into the incubator and giving it a relative air humidity of about 95%. This high air humidity made remoistening of the blotters during incubation unnecessary as they never dried out under these conditions.

Agar dishes were often first kept in darkness for colony formation, for which purpose a number of lockers were available in the transfer room. Afterwards they were transferred to the illuminated incubators.

2.3.2 *Transfer room and sterilizing equipment*

In a corner of the laboratory a transfer room had been constructed. In this room the air is purified from air-borne contamination by means of a Honeywell electronic aircleaner. Inside the agar tests were prepared as well as all experiments requiring a relatively germ-free environment. In front of the transfer room an electric oven was placed in which glass ware could be sterilized by dry heat. The contents could be emptied through a guillotine window from within the transfer room. Further details were published elsewhere (LIMONARD, 1967b).

For the sterilization of agar media, soil, etc., an autoclave was available.

2.3.3 *Greenhouse and climatic cells*

In the greenhouse some sections were available in which experiments could be carried out in tablets filled with soil. As it could not be cooled, it was only used in winter when a minimum temperature of around 15°C was obtainable.

Climate rooms were used for conditioned soil tests. These cells could be kept at any desired temperature between 7°C and 35°C. They were illuminated with horizontal fluorescent daylight tubes above the shelves. This weak illumination was sufficient for a reasonable normal seedling development. A difficulty was

that the relative air humidity inside was low, so that special measures, such as the use of polythene coverings, were necessary to restrict loss of moisture.

2.4 Use of scientific names

For practical reasons fungal pathogens have in this publication been indicated by their most commonly used scientific names. These are usually those pertaining to the imperfect state which is most frequently met in seed health testing work.
More recently adopted synonyms, names of perfect states and authors can be found in table 1.

3 BACTERIAL ANTAGONISM

3.1 Introduction, the 'wet blotter effect' (WBE)

The investigations here reported started with an earlier observation made by the Station's Health Testing Department, namely that tests for certain infections gave lower percentages when incubated on rather moist blotters than on rather dry ones (de Tempe & Limonard, 1965).

For convenience' sake this phenomenon was given the name of 'wet blotter effect', or WBE.

A part of the results of these investigations have already been published (Limonard, 1967a).

3.2 The prevalence and importance of the WBE

In order to determine the extent and limits of this phenomenon as many different seed-borne infections were drawn in as could be found. These infected seed samples were tested on blotters of three different moisture levels indicated as 'dry', 'normal' and 'wet' To obtain 'wet' blotters these were dipped into a tray with water and left in it sufficiently long to become thoroughly saturated, allowed to drip for a few moments and then placed into the trays. 'Dry' blotters were prepared by pressing 'wet' ones between dry (not moistened) blotters. The 'normal' ones were of a wetness more or less intermediate between the other two, and equal to the substrate moisture level used in the Station's routine tests. No attempt was made to standardize the substrate moisture more exactly, but determinations were made of the actual amount of water given. It

was found that in the 'dry', 'normal' and 'wet' objects this was respectively about 30, 45 and 60 ml of water per tray (white and grey blotter taken together, or 136, 205 and 273% of the blotter weight). The results of this survey are presented in table 2.

It should be remarked that the percentage figures of the above table do not give a complete picture of the WBE phenomenon. The fungi found on 'wet' blotters show a poorer growth, and the number of fungal structures found on infected seeds is much less. Even if sometimes the difference between the numbers of infected seeds found on 'wet' and 'dry' blotters are small, those on the 'wet' blotters may be harder to find for this reason. For the infections of table 2B showing no quantitative WBE, there was also no apparent qualitative WBE.

TABLE 2. The 'wet blotter effect' of different seed-borne infections

host seed	pathogen	number of samples	percentage of infected seeds in 3 blotter tests		
			'wet'	'normal'	'dry'
A. *infections showing WBE*					
Flax	*Botrytis cinerea*	28	2.3	4.8	7.9
Flax	*Ascochyta linicola*	7	38.1	42.0	53.6
Flax	*Alternaria linicola*	1	28.5	36.8	44.3
Lettuce	*Botrytis cinerea*	2	13.4	19.4	27.2
Carrot	*Stemphylium radicinum*	14	5.6	10.7	20.6
Carrot	*Alternaria dauci*	8	3.2	5.1	9.9
Radish	*Alternaria raphani*	3	12.3	26.7	31.5
Cabbage	*Alternaria brassicicola*	12	9.2	24.3	33.4
B. *infections not showing WBE*					
Wheat	*Fusarium* spp.	20	23.6	23.7	22.2
Barley	*Helminthosporium sativum*	10	27.1	28.6	27.7
Barley	*Helminthosporium* spp.*	14	23.0	22.4	21.7
Oats	*Helminthosporium avenae*	12	34.6	34.0	33.0
Ryegrass	*Helminthosporium* spp.**	8	23.2	24.7	26.3
Meadow fescue	*Helminthosporium* sp.***	3	18.4	18.2	19.3
Cabbage	*Phoma lingam*	7	4.5	4.8	4.6
Beets	*Phoma betae*	10	41.6	43.9	40.2
Spinach	*Colletotrichum spinaciae*	1	13.4	13.0	11.2

* Probably mainly *H. siccans*

** Mainly *H. siccans* and *dictyoides*

*** Probably *H. dictyoides*

3.3 Is the WBE a fungistatic or a fungicidal phenomenon?

One of the first questions arising was whether the WBE is a fungicidal or a fungistatic phenomenon. Therefore, among others, the following experiments were carried out with a flax seed sample heavily infected with *Botrytis cinerea.* Seed coats and cotyledons of this samples showing no fungal growth after incubation on 'wet' blotters, were plated out on potato dextrose agar in sterile petri dishes. This procedure did only occasionally result in the fungus still growing out from them. It was concluded that the 'wet' substrate partly kills the fungus and for a small part prevents it to develop.

Another experiment in this connection was the change of 'wet' blotter tests into 'dry' ones after various periods of time, and conversely. The change of 'wet' to 'dry' was carried out by blotting the 'wet' ones on filterpaper and putting a 'dry' thick grey blotter underneath it The change of 'dry' to 'wet' was carried out by dipping the bottom of the trays gently in water so that the blotters could soak up extra water. The results are given in table 3.

Table 3. Effect of changing the blotter moisture level during incubation on the percentage of *Botrytis cinerea* found in a flax seed sample

moisture regime	infection percentage found after		
	4 days	7 days	10 days
10 days 'wet'	5	17	17
7 days 'wet' then 3 days 'dry'	5	16	16
4 days 'wet' then 6 days 'dry'	5	18	19
2 days 'wet' then 8 days 'dry'	10	25	29
10 days 'dry'	17	29	30
4 days 'dry' then 6 days 'wet'	24	31	32
2 days 'dry' then 8 days 'wet'	11	25	27

Apparently the first few days are decisive.
On 'wet' blotters the full damage is done in about 4 days, while on the other hand only 2 days on 'dry' ones are needed to give practically the full infection percentage.

An interesting observation made with the sample of table 3 is moreover, that even when the seeds had been kept in water for 4 days at 20°C and were then transferred to blotters, still 4% of the original 30% infection had survived.

Yet another pertinent observation is the fact that in experiments with carrot

seeds placed on a piece of carrot leaf floating on water, occasionally *Botrytis cinerea* grew out of the seeds and grew excellenty on the water surface. Also in experiments with nutrition agar, which has a high water content – about 96% – the fungus grew submerged in the agar under conditions of low air humidity (brought about by means of saturated salt solutions). The water level itself, therefore, cannot be considered to be harmful to the fungus and to be the cause of the lower infection percentages found.

In one of the experiments made in connection with the topic of this section flax seeds were germinated on top of 25 g of oven-dry silversand in petri-dishes to which various quantities of water were added. It was found that at moisture levels just sufficient for seed germination *B. cinerea* did not or hardly develop. At somewhat higher moisture levels the fungus developed nicely but decreased again at higher moisture levels.

3.4 The relative importance of pathogen and seed in the WBE

The difference in sensitivity of seed infections to the WBE gave rise to the question, whether this is based on differences between the pathogens or between the seed species. This was studied by examining the behaviour of the same fungus in different kinds of seed. Natural infections of *Botrytis cinerea* were found in seeds of flax, lettuce, corn-salad, wheat and sunflower. Non-infected seeds of flax and lettuce were moreover artificially infected by shaking them in petri-dishes with a sporulating colony of the fungus, resulting in 100% surface infection.

Table 4. Influence of the kind of seed on the WBE for *B. cinerea*

host seed	number of samples	percentage of *B. cinerea* found on	
		'wet' blotters	'dry' blotters
A. *natural infections*			
flax	3	12.4	21.3
lettuce	2	11.1	23.8
corn-salad	7	31.0	30.4
wheat	2	10.3	11.0
sunflower	1	34.3	35.0
B. *artificial infections*			
flax	1	0	100
lettuce	1	0	100

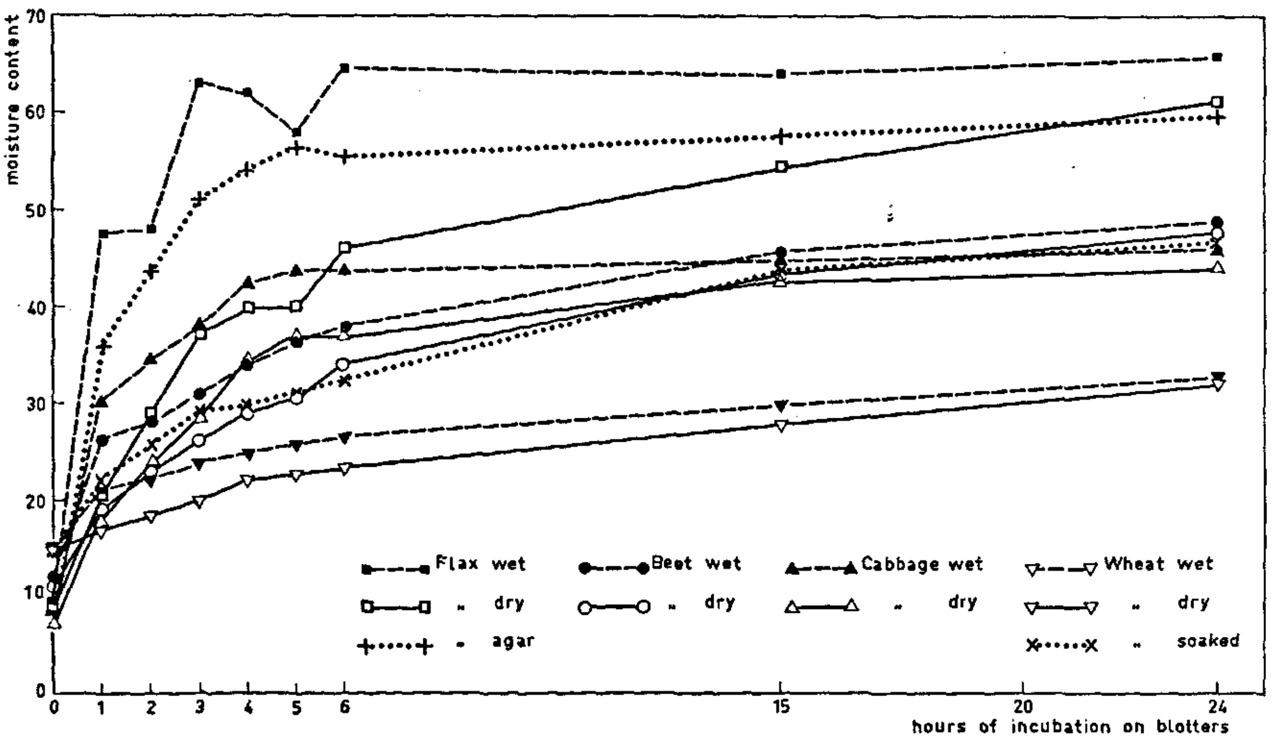


FIG. 1. Moisture percentages of seeds of flax, cabbage, wheat and beet, after various periods of incubation on 'wet' and 'dry' blotters. For flax seed also on malt extract agar and for wheat seed after soaking in water

in reaction of *Phoma valerianellae* in whole and in isolated true seeds points in the same direction.

3.6 The soak effect (SE) for seeds without WBE

The results of section 3.5 suggest that the speed of water uptake is an important factor involved in the WBE. It was therefore investigated whether by speeding up the rate of uptake by soaking the seeds in water a decreased percentage of infected seeds would be obtained in case of infections not showing the WBE. To this end 400 wheat seeds of two samples were put into 250 ml glass beakers and covered with a thin layer of tap-water. This amounted to about 40 ml of water per 400 seeds. After the soaking period the seeds were incubated on blotters to determine the percentage of *Fusarium* in them. It appeared that soaking for 24 hours in water of 20 °C decreased the percentage of *Fusarium* found, while soaking in water of 10 °C did not, as compared with non-soaked controls (table 6).

Table 6. Effect of soaking for 24 hours in water at 10 and 20 °C on the percentages of *Fusarium* spp. obtained in blotter tests with 2 wheat seed samples

sample	control	10 °C-soak	20 °C-soak
1	35	32¼	17¾
2	26¼	25¾	6¼

The soaking water of 20 °C became cloudy and malodorous. This indicated that bacteria might be implicated. It was then investigated whether the addition of the antibiotic terramycin to the water would have any influence on the soak effect. At the same time the occurrence of a SE for other infections without a WBE was investigated.

Table 7 gives the results of soak experiments in water with 100 p.p.m. terramycin for two seed samples of wheat, oats, barley and beet. These data are a confirmation of the idea that bacteria are involved. Furthermore these results led to the conclusion that for seeds taking up water more slowly soaking in water of 20 °C has the same effect as a 'wet' blotter on seeds taking it up at a faster rate.

TABLE 7. Effect of soaking for 24 hours in water of 10° and 20°C, and in a 100 p.p.m. terramycin solution at 20°C, on the percentages of different fungi found in blotter tests

sample		pathogen	control	soaked for 24 hours in		
				water of 10°C	water of 20°C	terramycin sol'n of 20°C
Wheat	1	*Fusarium* spp.	35	33½	17¾	33½
	2	*Fusarium* spp.	26	23¾	6¾	25¼
Oats	1	*Helminthosporium avenae*	34½	31	17¾	28¼
	2	*Helminthosporium avenae*	18½	19¼	11	16¾
Barley	1	*Helminthosporium* spp.	22¼	21½	16	20½
	2	*Helminthosporium* spp.	18½	17	8¼	17
Beet	1	*Phoma betae*	61½	58¾	40¼	65¾
	2	*Phoma betae*	39½	45¼	21	43

3.7 THE INFLUENCE OF TEMPERATURE ON THE WBE

The results mentioned in section 3.6 suggested a similarity between SE and WBE. They also indicated that bacteria were involved. Hence the possibility of bacterial antagonism as the cause of the WBE was explored by performing 'wet' and 'dry' blotter tests for *B. cinerea* in flax seed at a temperature of 12°C, instead of the usual 20°C. A temperature of 12°C was chosen as seed germination and seedling development were then still sufficient. Another reason was the fact that at the time it was difficult to have an illuminated incubator run at a still lower temperature.

The results given in table 8 show that the WBE is still very evident at 12°C for this infection. The temperature effect as found for the soaking of wheat seed apparently does not apply here. NEWHOOK (1951) reported that bacterial antagonism against *B. cinerea* could still be found at 4°C. The reason for the diffe-

TABLE 8. Effect of temperature on the WBE for *Botrytis cinerea* in flax seed – average percentages for 10 samples

temperature and duration of incubation	on 'wet' blotters	on 'dry' blotters
12°C		
after 8 days	3.3	5.0
after 11 days	8.4	12.2
20°C		
after 8 days	9.0	20.1

rence in reaction to temperature between the WBE for *B. cinerea* in flax seed and the SE for *Fusarium* spp. in wheat seed is probably due to the much slower rate of *Botrytis* development at low temperature. This is shown by the lower percentages obtained for this pathogen at 12°C as compared with 20°C, even after a longer period of incubation.

Fusarium nivale, on the other hand, which was the most important *Fusarium* species present in the wheat samples used, is stimulated by lower temperatures in blotter and soil tests (cf. section 5.2).

3.8 The influence of antibacterial antibiotics on WBE

In analogy with the soak experiments of section 3.6, tests for *Botrytis cinerea* in flax seed were carried out in which the blotters had been moistened with solution of the antibacterial antibiotics terramycin and streptomycin. The results of such an experiment are given in table 9. These showed that the WBE can be counteracted by the above antibiotics. The data were confirmed many times in later experiments (cf. Fig. 2).

Table 9. Effect of adding antibacterial antibiotics to 'wet' blotters on the results of the blotter test for *Botrytis cinerea* in 3 samples of flax seed

testing conditions	percentage of *B. cinerea* found in sample:			
	1	2	3	average
'dry' blotter	13½	34¾	11¼	19.8
'wet' blotter	7¾	19¾	6¾	11.4
'wet' blotter with 50 p.p.m. terramycin	15	31	12	16
'wet' blotter with 100 p.p.m. terramycin	10¾	36½	12¼	19.8
'wet' blotter with 100 p.p.m. streptomycin	14	30¾	14¼	19.7
'wet' blotter with 50 p.p.m. terramycin and 100 p.p.m. streptomycin	17	34	13¼	21.4

It was concluded that the addition of antibiotics to blotter tests would remove the factor substrate moisture as a cause of fluctuation of the results.

3.9 Experiments with stimulation of bacteria

In order to further consolidate the bacterial antagonism hypothesis for the WBE, the following reasoning was used. If suppression of bacterial growth

(antibiotics) reduces or eliminates the WBE, stimulation of bacterial growth will increase it. To this end sterile Bacto Nutrient Broth (Difco) with and without a rough bacterial culture isolated from flax seeds was added to the blotters. This reduced the *B. cinerea* percentage to zero in all cases tried. During the incubation of such tests ammonia could be smelled. The remarkable strong effect of the sterile broth without bacteria has to be explained by stimulation of the growth and development of the natural bacterial seed flora.

3.10 Experiments on the influence of pH

Another way of trying to differentially stimulate bacterial and fungal development in the blotter test was by means of pH regulation. The pH of the tapwater ordinarily used was 6.9–7.0. By means of paper pH indicator strips it was found, that the pH of the blotters remained at this level during the incubation period.

By the use of 0.1 M Sörensen phosphate buffer solutions instead of ordinary water, the pH of the blotters was increased or decreased. The experiments were carried out on miniature Copenhagen tables, the blotters being kept wet by means of paperstrips hanging in trays filled with water or buffer-solution.

The treatments used were wet blotters with pH 5.2, pH 8.0, tapwater (pH (pH 6.9–7.0) and 'dry' blotters (also pH of tapwater). The 'dry' blotters were of course without paper wicks. The results of this experiment are given in table 10. If the buffer-solutions were added in normal blotter tests similar results were obtained.

Table 10. Effect of pH of blotter moisture on the percentages of *Botrytis cinerea* found in flax seed on blotters on miniature Copenhagen tables, and on 'dry' blotters, both inside incubators

sample	tapwater (pH 7.0) 'dry'	buffer solution pH 5.2 'wet'	tapwater (pH 7.0) 'dry'	buffer solution pH 8.0 'wet'
1	14¼	10	5¾	¼
2	33	28¼	17¾	10¼
3	10¾	9¼	6	1¼

It is known that *B. cinerea* is injured by high pH (Newhook, 1951). Therefore such an experiment was also carried out with a carrot seed sample infected with *Stemphylium radicinum.* Table 11 gives the results of that experiment, in which the effect of the addition of Difco Bacto Nutrient Broth with and without a rough bacterial culture isolate from carrot seed was also tested. It can be

TABLE 11. Effect of bacterial antagonism in the blotter test for *Stemphylium radicinum* infection of carrot seed as shown by the results of an experiment with one sample

blotters moistened with	'wet'	'dry'
tapwater	37¼	46¾
P-buffer, pH 8.0	35¾	47⅛
Difco Nutrient Broth	26⅛	49
Idem, but with rough bacterial culture from carrot seed added	20	–
P-buffer, pH 5.2	60¼	70¼
100 p.p.m. terramycin solution	63	69¼

seen that for this infection the results were quite similar, except for the fact that here the addition of Nutrient Broth and of bacteria did not reduce the percentage as strongly as for *B. cinerea* in flax seed.

In the above test, table 11, the first 4 'wet' conditions gave much development of *Cephalosporium roseum* and not of other fungi. In the last 2 'wet' and in the 'dry' treatments hardly any *C. roseum* was found, but the development of many other fungi was markedly increased *(Fusarium, Alternaria, Epicoccum, Stachybotrys)*. It can be remarked here that the maximum percentage found for *Stemphylium radicinum* was around 70% and that this can be reached in various ways.

3.11 Blotter experiments with malt extract and Brestan

Many Ditch flax seed lots are exported to France and these lots are tested for *B. cinerea* with the blotter method in the Netherlands, whereas the results of this screening were occasionally checked by the French official seed testing station by means of the agar method. For that reason the findings concerning the WBE were passed on to Paris.

The blotter method is known to produce lower figures for this infection than the agar method, but when the blotter moisture content was kept low, the differences became smaller, or even negligible (section 3.12). The French workers then tried the addition of 3% malt extract to the blotters as a possible way of increasing the percentages found to the level of the agar method. They succeeded in doing this (ANSELME *et al.*, 1965) and their results may now be explained by the low pH of the malt extract, which stimulates fungi but is less suitable for bacteria. Their experiments were repeated by us with similar results (table 12).

The French investigators found it necessary to check the growth of sapro-

TABLE 12. Effect of the addition of 0.1 % Brestan and 3 % Difco Malt Extract to blotters on the average percentage of *Botrytis cinerea* found in 5 flax seed samples

testing conditions	*B. cinerea*
'dry' blotters	16.8
'wet' blotters	8.9
'wet' blotters with Brestan	16.0
'wet' blotters with malt extract	17.5
'wet' blotters with malt extract and Brestan	17.6

phytes by the addition of 0 1 % Brestan (20 % triphenyl tinacetate) to the malt extract. With the samples we used and under our conditions this did not prove to be necessary. As can be seen in table 12 the addition of Brestan to the blotters seemed by itself to have already some effect on the WBE. The apparent tolerance of *B. cinerea* to Brestan did not apply to other pathogenic fungi tested in this respect, viz. *Stemphylium radicinum* in carrot seed and *Alternaria brassicicola* in cabbage seed. Moreover it was found to be toxic to the flax seedlings, the roots of which were very much stunted, so that the plants often fell over Host vigour may therefore also play a role in this case (ch. 4). It may be of interest that KAARS SIJPESTEIJN *et al.* (1962) reported triphenyl tin acetate to be especially toxic to Gram-positive bacteria and less to *Botrytis allii*.

The addition of Duter (triphenyl tin hydroxide) was also tried, but although this was apparently not very toxic to *B. cinerea* it did not increase the percentages in 'wet' blotter tests either.

3.12 EXAMINATION OF THE DIFFERENCE BETWEEN BLOTTER AND AGAR TEST RESULTS FOR BOTRYTIS CINEREA IN FLAX SEED

It is well known that bacteria in general prefer higher pH values than fungi. Part of the differences obtained (cf. sections 3.10, 3.11) may therefore be due to this factor. For many infections lower percentages are obtained on blotters than on agar. An interesting case is that of *B. cinerea* infection in flax seed. The results of agar tests for this infection are, as a rule, higher than those obtained by the blotter method (DE TEMPE, 1958, 1961, 1963; VAN DER SPEK, 1965). Up till now this was explained 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 must attack the young seedlings in order to be counted. When the existence of the WBE and the nature of its cause became known the question arose as

to how the percentages obtained in blotter test with low substrate moisture or antibiotics, would compare with those of agar tests. To this end 31 flax seed samples were tested in four ways, viz. on 'wet' blotters, on 'dry' blotters, on 'wet' blotters with 100 p.p.m. terramycin, and on malt extract agar. The four tests were carried out simultaneously for the same sample, so that any decrease of the infection due to ageing of the samples did not play a role. The results of these determinations are given in Fig. 2.

These data clearly show that the results of the blotter tests in which the bac-

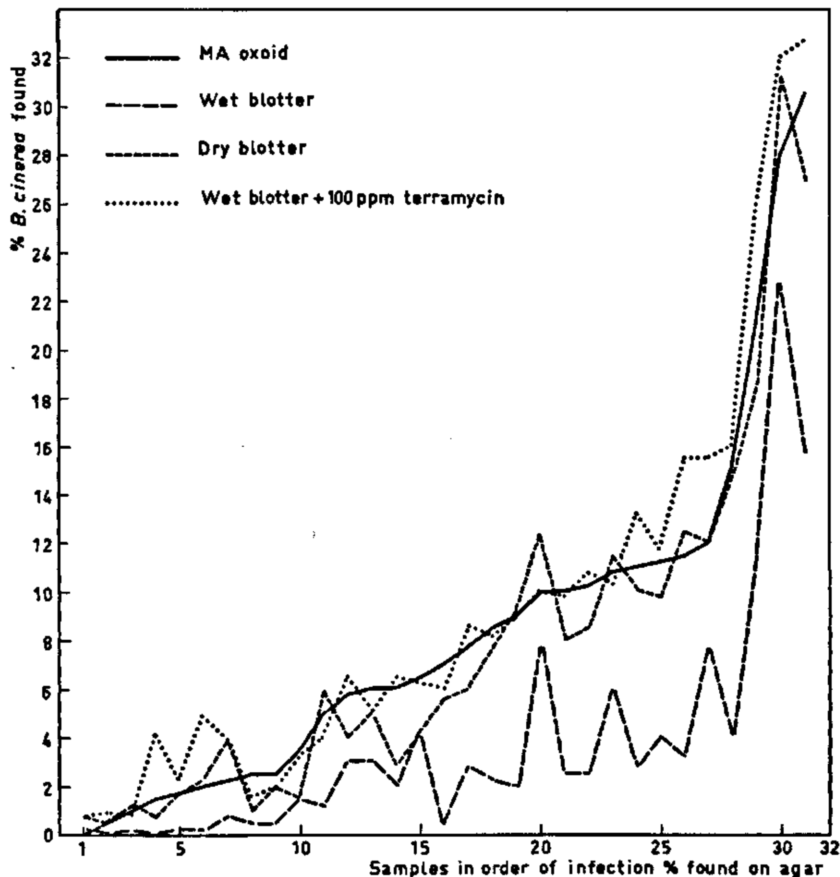


FIG. 2. Percentages of *Botrytis cinerea* found for 31 samples of flax seed tested on malt extract agar, on 'wet' blotters, on 'dry' blotters and on 'wet' blotters with 100 p.p.m. terramycin. The samples have been arranged in order of increasing *B. cinerea* percentage found in the agar test

terial development has been checked, are as high as those obtained with the agar method.

The conclusion is that apparently the reason for the difference between the agar and blotter test results could be found in the lesser importance of bacterial antagonism in the agar test. It should be noted that flax seeds are normally not surface-disinfected before plating on agar. It is not a necessity and their slimy seed coat would cause difficulties. That notwithstanding bacterial antagonism is not important in the agar test is now thought to be due to pH and moisture level. The moisture uptake of seeds on malt extract agar is intermediate between that on 'dry' and 'wet' blotters, and the pH of the malt axtract agar (Oxoid) used was 5.4 (cf. with influence blotter pH, section 3.10).

Agar media normally contain some 96% water, but this water is fixed and the agar surface is rather dry. It was also examined whether increasing the physiological 'wetness' of the agar medium would lower the percentage of the infection obtained. This was done by incubating the seeds on PDA with agar percentages of 2, 1, ½ and ¼. The medium with ¼% agar was practically fluid and the seeds had some tendency to float around. The results given in table 13 show that only when the medium does not solidify (below ½% agar) the *B. cinerea* percentages appearing from flax seeds are reduced. It was furthermore observed that at this lower agar concentration more bacterial growth occurred.

When flax seed was incubated on nutrient agar (Difco) of pH 6.8 with and without terramycin, the influence of bacterial antagonism was very great (table 14).

TABLE 13. Effect of agar concentration of potato dextrose agar on the percentage of *Botrytis cinerea* found in 3 flax seed samples

sample	agar concentration			
	2%	1%	½%	¼%
1	27¼	27½	25½	11
2	36¼	38¼	29	27¾
3	20¾	19½	17	5¼
average	28	28	24	14

It has already been stated, that *B. cinerea* is hampered by a high pH. When it starts growing it will acidify the medium by its metabolic products (NEWHOOK, 1951). Hence it will probably be a matter of which is first to begin growth, the bacteria or the fungus.

TABLE 14. Effect of the addition of terramycin to Difco Bacto Nutrient Agar on the percentage of *Botrytis cinerea* found in flax seed samples

Sample	without terramycin	with 15 p.p.m. terramycin
1	14	22
2	7½	16½

3.13 EXPERIMENTS ON THE WBE FOR SOME OTHER INFECTIONS

In the foregoing sections it became clear that the WBE as well as the SE can be explained in terms of bacterial antagonism. Most of the experiments described were carried out with *B. cinerea* in flax seed. When other infections showing WBE were tested in similar ways, the same results were obtained. This is illustrated in table 15, which gives the testing results for a number of such infections on 'dry' and 'wet' blotters and on 'wet' blotters with 100 p.p.m. terramycin. It should be remarked again that the figures given do not present the entire picture. The quantity of structures of the pathogen per seed was much lower on 'wet' blotters than on 'dry' ones. Hence the recognition of infected seeds on 'dry' blotters and on those with terramycin is much easier than on 'wet' ones.

TABLE 15. Effect of the addition of 100 p.p.m. terramycin to 'wet' blotter tests on the average percentages found for some infections showing WBE

infection	number of samples tested	'wet'	'dry'	'wet' with terramycin
Ascochyta linicola (flax)	7	5.7	10.3	15.7
Alternaria linicola (flax)	1	21.3	41.3	43.5
Alternaria brassicicola (cabbage)	12	50.2	62.5	59.1
Stemphylium radicinum (carrot)	12	19.7	24.9	25.6
Alternaria dauci (carrot)	5	7.1	13.4	14.1
Alternaria raphani (radish)	2	13.4	27.6	25.9

3.14 THE INFLUENCE OF THE ADDITION OF TERRAMYCIN ON THE BLOTTER TEST RESULTS FOR FUSARIUM SPP. IN WHEAT SEED

The question could be asked whether the addition of terramycin in blotter tests for infections without WBE might have any effect. This was tried for a number

of samples of such infections without any apparent effect on the percentages obtained, as can be seen in table 16. It was observed, however, that in the case of the *Fusarium* infection of wheat seed there was a shift from light to severe symptoms. The percentage of severe symptoms was increased with an amount equal to that with which the percentage of light symptoms was decreased (table 17).

TABLE 16. Effect of the addition of 100 p.p.m. terramycin to 'wet' blotters on the average percentage found for some seed infections showing no WBE

infection	number of samples	without terramycin	with terramycin
Fusarium spp. (wheat)	10	31.3	31.6
Helminthosporium avenae (oats)	8	35.6	34.3
Helminthosporium spp. (barley)	4	18.0	18.5
Phoma betae (beet)	4	43.3	44.1

TABLE 17. Effect of the addition of 100 p.p.m. terramycin solution to blotters on the percentages of symptoms in testing for *Fusarium* spp. in the wheat seed samples of table 16

blotter test symptoms	without terramycin	with terramycin
dead seeds with *Fusarium*	1.8	1.9
severe symptoms	6.2	11.4
light symptoms	23.4	18.4
total *Fusarium*	31.3	31.6

These results resemble those reported in section 5.2 on the effect of lower temperature in blotter tests for this infection. They were taken as an indication that bacterial antagonism might also play some role in ordinary blotter tests for *Fusarium* in wheat seed.

3.15 Additional experiments on the SE with wheat seed and the isolation of one of the implicated bacterial species

Subsequently to the experiments and results reported in section 3.6 a larger number of soak experiments was carried out to examine this phenomenon in

further detail. In table 18 the data of experiments on the influence of duration of the soaking on the soak effect are given for a weat seed sample at 10 °C and 20 °C. It is clear that short soaking periods have but little consequence and that a certain duration is necessary for any effect. The SE at 10 °C was smaller and started later. All subsequent experiments were therefore carried out at 20 °C and usually for a duration of 24, or sometimes 48, hours.

TABLE 18. Effect of duration of the water soak at 10 and 20 °C on blotter test percentages of *Fusarium* subsequently found in a wheat seed sample

hours of soaking	at 10 °C	at 20 °C
0	32½	32½
1	33½	33
6	34	33½
16	32	34
24	31	24
48	21½	10
72	13½	3½

TABLE 19. Effect of soaking for 24 hours in water and 100 p.p.m. terramycin solution on the average percentages of symptoms obtained in subsequent blotter tests for *Fusarium* spp. in 26 wheat seed samples

blotter test symptoms	non-presoaked control	water-soak	terramycin soak
dead seeds with *Fusarium*	3.1	2.0	3.9
severe symptoms	8.3	5.1	10.8
light symptoms	23.3	16.4	19.8
total *Fusarium*	34.6	23.5	34.6

The SE and its nullification by terramycin was further confirmed by the testing in blotters of 26 wheat samples carrying *Fusarium* infection after soaking respectively in water and 100 p.p.m. terramycin solution at 20 °C, and of the unsoaked control. This gave the data of table 19. It has to be remarked that in this case soaking of wheat seeds in terramycin solution gives a shift from light to severe symptoms as well as an increase in the number of dead seeds with *Fusarium*. The latter increase was correlated with a corresponding decrease in percentage of ungerminated (dead) seeds without *Fusarium*.

Apparently the fungus can do greater damage after the terramycin soak of the seed. When the concentration of the terramycin solution was increased, the effect was even more pronounced. This is shown by the data of table 20, where the results of an experiment are given in which 5 wheat samples were soaked during 24 hours in water, 100 and 200 p.p.m. terramycin solution.

To check whether other antibiotics than terramycin would yield similar results, two wheat seed samples were soaked in 100 p.p.m. and 200 p.p.m. of each terramycin, streptomycin and chloramphenicol. The results are given in table 21. They show that there is no real difference between the three antibiotics in their action.

The implication of bacteria in the SE was further examined by the soaking of wheat seeds in sterile nutrient broth instead of water. This turned out to speed up the SE significantly, as can be seen from the data of table 22 which gives

TABLE 20. Effect of soaking for 24 hours in water and in 100 and 200 p.p.m. terramycin solution on the average percentages of symptoms obtained in subsequent blotter tests for *Fusarium* spp. in 5 wheat seed samples

blotter test symptoms	non-presoaked control	water-soak	terramycin-soak 100 p.p.m.	terramycin-soak 200 p.p.m.
dead seeds with *Fusarium*	2.7	2.8	4.1	5.5
severe symptoms	3.2	2.1	4.0	5.2
light symptoms	17.0	8.4	15.8	16.1
total *Fusarium*	22.9	13.3	23.9	26.8

TABLE 21. Effect of soaking in water and various antibiotic solutions for 24 hours at 20 °C on the average percentages of symptoms obtained in subsequent blotter tests for *Fusarium* spp. in 2 wheat seed samples

	dead seeds with *Fusarium*	severe symptoms	light symptoms	total *Fusarium*
non-presoaked control	2	8¾	37	47¾
water	1⅛	2¾	18¼	22⅛
100 p.p.m. terramycin	2¾	10¾	30¼	43¾
100 p.p.m. streptomycin	2⅛	5⅛	32¼	40¼
100 p.p.m. chloramphenicol	2¼	8⅛	29¼	40
200 p.p.m. terramycin	4½	10⅛	31¾	46¾
200 p.p.m. streptomycin	1¾	10	35⅛	47¼
200 p.p.m. chloramphenicol	2¼	7¼	34¼	43¾

the averages for five samples. The conclusion was, that the nutrient broth stimulated bacterial growth and thereby the SE. If a little (about ½ ml) of this medium in which seeds had been soaked at 20°C for 24 hours, was taken and added as inoculum to the water of another soak experiment this also gave an increased SE (table 23).

In order to see whether soil bacteria would give a similar effect, some greenhouse soil was mixed with water and the seeds were soaked in the resulting soil suspension. This also gave a significant reduction in percentage of *Fusarium* obtained (table 23).

From the results of the experiments discussed above it became abundantly clear, that bacterial antagonism is also involved in the SE.

TABLE 22. Effect of soaking in water and in Difco Nutrient Broth for 24 hours at 20°C on the average *Fusarium* percentage found in subsequent blotter tests in 5 wheat seed samples

soak-treatment	*Fusarium*
non-presoaked control	55.2
water	38.3
nutrient broth	17.4

TABLE 23. Effect of soaking in water, water with ½ ml of inoculum from a previous soak experiment, and in greenhouse soil suspension for 24 hours at 20°C on the average *Fusarium* percentage found in subsequent blotter tests for 2 wheat seed samples

soak-treatment	*Fusarium*
non-presoaked control	54
water	34¾
water with inoculum	21¾
soil-suspension	7½

3.16 MASS AND INDIVIDUAL SOAK EXPERIMENTS AND THE OCCURRENCE OF POSITIVE SOAK EFFECTS

It was frequently observed, that the soaking of wheat seeds caused a much faster decrease of the infection in certain samples than in others. This was thought to be due to one or both of two reasons. The first was the ratio between super-

ficially and more deeply seated infection, the superficial infection being considered the more sensitive one (cf. section 3.4, ch. 6).

The second reason might be quantitative and qualitative differences in the bacterial floras of the samples.

To study the latter possibility two wheat seed samples were selected with a similar *Fusarium* load, but differing in their SE. In the following they have been indicated sample A and B. Table 24 and 25 give their *Fusarium* percentages and table 24 in addition the SE. When from both samples 400 seeds were plated out on nutrient agar, sample A gave rise to bacterial colonies from 61 % of the seeds and sample B from 38 % of the seeds. A difference that was considered even more important, was that about two thirds of the bacterial colonies of sample A were of a spreading type, but in sample B only one eighth. Among the spreading types of bacteria one kind was quite frequent and easily distinguished from the others by its characteristic growth on nutrient agar. It grew very rapidly, frequently covering the entire plate in 24 to 36 hours at 20 °C. The colony was greyish-white in colour with a rhizoid growth. The organism was a sporeforming, Gram-positive small rod. It was isolated and used in some later experiments with artificial bacterization of seeds (section 3.22). Later surveys showed it to be quite frequently present on many kinds of seeds. It was identified by Ir. J.

TABLE 24. Percentage of *Fusarium* infection for the 2 wheat seed samples A and B (cf. text) as determined on potato dextrose agar

sample	without NaClO pretreatment			with NaClO pretreatment		
	F. nivale	*F. graminearum*	total *Fusarium*	*F. nivale*	*F. graminearum*	total *Fusarium*
A	29	5	36	30	2	32
B	26	0	28	29	2	32

TABLE 25. Effect of soaking in water of 20 °C for various lengths of time on the *Fusarium* percentages obtained in subsequent blotter tests for the 2 wheat seed samples A and B (cf. text and table 24)

sample	hours of soaking				
	0	16	24	48	72
A	41	39	21	9	11
B	39	42	33	17	13

VAN DER TOORN (Lab. of Microbiology, Technical University, Delft), as *Bacillus cereus* var. *mycoides* (FLÜGGE) SMITH, GORDON & CLÜRK. (Plate I, Fig. 2).

The greater abundance of bacteria and especially of those with a spreading type of growth in sample A, was thought to be related to the stronger SE of that same sample. It was realized that mass soaking, such as carried out thus far, might result in an easy spread of bacteria from seed to seed in the soaking water. Therefore some experiments were carried out with these two samples in order to see, whether it would make any difference if the seeds were soaked individually instead of together. Individual soaking took place in small glass vials with 0.2 ml of liquid given per seed. This was about twice as much as that used in mass soaking. Using more water per seed can be assumed to dilute the bacteria and the nutrients available to them and hence to decrease their effect. This was indeed found to be the case. If proportionately large quantities of liquid were added, hardly any SE could be observed. For technical reasons it was difficult to use less than 0.2 ml of water per seed in individual soaking.

In the mass soaked controls 80 ml – instead of the usual 40 ml – of water per 400 seeds was used. The results are given in table 26.

It can be seen, that whereas for sample A after 48 hours the results of individual and mass soaking ran more or less parallel, that for individual soaking of sample B after the same time was quite different. For sample B another, ear-

TABLE 26. Effects of mass and individual soaking in 3 different liquids for 24 and 48 hours at 20°C on the *Fusarium* percentages obtained in subsequent blotter tests for the 2 wheat seed samples A and B (cf. text and tables 24 and 25)

sample	soak-treatment mass or individual soaking	soak liquid	length of soaking	
			24 hours	48 hours
A	mass	water	19½	9½
		200 p.p.m. terramycin	42	39½
		nutrient broth	11	4½
	individual	water	24½	10½
		200 p.p.m. terramycin	42	51
		nutrient broth	12½	7
B	mass	water	35	21½
		200 p.p.m. terramycin	44½	43
		nutrient broth	15	6
	individual	water	41	47
		200 p.p.m. terramycin	88	87
		nutrient borth	26½	25

lier, experiment with seeds sealed in small polythene bags with the same soak-king liquids had given practically identical results. Hence it was concluded, that the influence of the bacterial population per seed was quite important. This was further proved by running an experiment in which 100 seeds of sample B were rubbed over the surface of *B. cereus* var. *mycoides* colonies on nutrient agar. These seeds were then soaked individually for 24 hours and this resulted in a *Fusarium* percentage of as low as 7 in a subsequent blotter test.

The fact that soaking of seeds may also increase the percentage of infection found was observed with 5 samples of corn salad seeds infected with *Phoma valerianellae*. These seeds yielded an average of 5¼% after 9 days on blotters at 20°C and with NUV light. If tested in the same way after mass soaking in water of 20°C for 24 hours, they yielded an average of 31½%. Such a positive SE was also found by IKENBERRY (1961) for *Fusarium moniliforme* infection of corn seeds.

Hence it was concluded that soaking has two effects on fungal infections on seeds, namely

A. a negative effect, caused by the stimulation of bacterial growth and thus by increase of antagonism
B. a positive effect, caused by the stimulation of fungal growth.

In this publication with 'soak effect' is always meant the negative one, unless otherwise stated.

3.17 THE INFLUENCE OF AERATION ON THE SE

IKENBERRY (1961) in his soaking experiments with corn seeds infected with *F. moniliforme* also used aerated water. This gave a higher positive SE than non-aerated water (cf. 3.16). In order to examine the effect of aeration on the SE obtained in our experiments with wheat seeds, some experiments were carried out in aerated water. Aeration of the soak water was accomplished by means of a small aquarium pump. The results are given in the tables 27 and 28, and show that the aeration practically eliminated the SE. This was difficult to explain. Possibly fungitoxic products are produced by the bacteria involved especially under anaerobic conditions.

Another observation on the SE is still to be made. Most of the soak experiments here described were of a duration of 24 and sometimes of 48 hours. During this period no harmful effects on germination of the seeds were observed. If the soak period was still further prolonged, however, the germination percentage started to decrease. This decline in germination capacity was also coun-

Table 27. Effect of aeration during soaking in water for 24 hours at 20°C on the average percentages of symptoms obtained in subsequent blotter tests for *Fusarium* in 4 wheat seed samples

blotter test symptoms	soak-treatment		
	non-presoaked control	non-aerated water	aerated water
dead seeds with *Fusarium*	1.8	0.3	2.0
severe symptoms	3.0	2.2	4.3
light symptoms	32.2	15.0	30.2
total *Fusarium*	37.0	17.5	36.5

Table 28. Effect of aeration and addition of 100 p.p.m. terramycin on the soak-effect in water of 20°C, as exemplified by the average percentage of symptoms obtained in subsequent *Fusarium* blotter tests with 2 wheat seed samples

soak-treatment	blotter test symptoms				
	hours of soaking	dead seeds with *Fusarium*	severe symptoms	light symptoms	total *Fusarium*
control	0	0.5	4.1	39.1	43.7
non-aerated water	24	0	2.5	24.2	26.7
aerated water	24	1.0	3.2	38.4	42.6
100 p.p.m. terramycin	24	0.8	5.8	36.1	42.7
non-aerated water	48	0	0.3	12.0	12.3
aerated water	48	0	2.2	38.4	40.6
100 p.p.m. terramycin	48	0.7	5.7	35.5	41.9

Table 29. Effect of soaking for 144 hours at 20°C in aerated or non-aerated water and in 200 p.p.m. terramycin solution on percentage of symptoms and seed germination obtained in subsequent *Fusarium* blotter tests in a wheat seed sample

blotter test symptoms	soak-treatment			
	non-presoaked control	non-aerated water	aerated water	terramycin solution
dead seeds with *Fusarium*	4½	1	4½	9
severe symptoms	1½	0	1	6½
light symptoms	33½	½	5½	12½
total *Fusarium*	39½	1½	11	28
abnormal seedlings	11	5½	9	10
non-germinated seeds	2½	74½	15	15½

teracted by aeration, as well as by terramycin. This can be seen in table 29, which gives the results of an experiment in which the seeds were soaked at 20 °C for 144 hours.

3.18 The influence of soaking on agar test results

When soaked wheat seeds are plated out on agar without antibacterial antibiotics, the result is always an abundant bacterial development around the seed and a very much reduced fungal growth. If such seeds are, however, plated out on agar to which 25 ppm terramycin has been added, the SE is no longer observed. In case the seeds have been soaked in terramycin solution the percentage of *Fusarium nivale* obtained on agar is often decreased. The reason for this reduction is the increased development of other fungi and of *F. graminearum*, which prevents *F. nivale* from being observed. The above is demonstrated by the data of table 30 and 31.

In many cases it is impossible to obtain agar test results for terramycin-soaked seeds due to a profuse development of *Mucor* spp., especially after longer

Table 30. Effect of soaking in water and 200 p.p.m. terramycin solution for 24 hours at 20 °C on the percentages obtained for *Fusarium* in subsequent blotter tests (A), and on those obtained for two *Fusarium* species and the total percentage of other fungi (without *Mucor* and *Penicillium*) in subsequent agar tests (B)

A. blotter method

sample	non-presoaked	water-soak	teramycin-soak
1	66	41	59½
2	15½	7	14½
3	69	48½	69½
4	46	41½	52
5	26½	19	33½

B. agar method without NaClO pretreatment (7 days at 20 °C on PDA)

sample	*F. nivale*	*F. graminearum*	other fungi	*F. nivale*	*F. graminearum*	other fungi	*F. nivale*	*F. graminearum*	other fungi
1	35½	3½	63½	27	4½	58½	15	11	96½
2	4½	2½	95	4	4	108	2½	7	104
3	27½	7½	90½	24½	9½	96½	19	12	110
4	22	6	124	23½	7	124½	9	9½	135
5	12	0	91½	13½	0	109	5½	0	119½

TABLE 31. Effect of soaking in water and 200 p.p.m. terramycin solution for 24 and 48 hours at 20 °C on the percentage of *Fusarium* found in subsequent blotter tests and of *Fusarium* spp., *Epicoccum* and *Alternaria* in subsequent agar tests on PDA (containing 25 p.p.m. terramycin) with and without NaClO pretreatment in a wheat seed sample

soak treatment	hours of soaking	with or without NaClO	blotter test for *Fusarium*	agar-test					
				F. nivale	*F. graminearum*	*F. avenaceum*	total *Fusarium*	*Epicoccum*	*Alternaria*
control	0	-	57½	31	6	3	40	38	24
control	0	+	-	35	3	2	41	30	15
water	24	-	41½	27	4	7	42	47	25
water	24	+	-	6	3	2	14	16	15
terramycin	24	-	59	15	12	0	27	53	27
terramycin	24	+	-	24	4	1	29	9	15
water	48	-	19½	25	6	8	39	43	23
water	48	+	-	6	2	1	9	17	12
terramycin	48	-	49½	11	11	1	23	64	36
terramycin	48	+	-	21	4	0	25	14	15

periods of soaking. In a later chapter (ch. 5) the introduction of a selective agar medium for the isolation of *Fusarium* spp. from seed will be described. On that medium, which also contains terramycin, only species of *Fusarium* can grow out, whereas *F. nivale* can be easily distinguished from the other species. This agar medium was then used for testing wheat seeds soaked in water and 100 ppm terramycin solutions at 20 °C for various periods of time. Table 32 gives the data of a representative experiment.

TABLE 32. Effect of soaking of a wheat seed sample for various periods in water or 200 p.p.m. terramycin solution on the percentages of *Fusarium* subsequently found after plating on a special selective *Fusarium* medium (peptone-PCNB agar) and also by subsequent blotter tests

soak-treatment	*Fusarium* blotter test	peptone-PCNB agar	
		F. nivale	other *Fusarium* spp.
non-presoaked control	56	31	41
24 hours in water	48	*	100
24 hours in terramycin	72	*	100
48 hours in water	19	*	100
48 hours in terramycin	63	*	100
72 hours in water	20	*	100
72 hours in terramycin	50	*	100

* about 30% underneath colonies of other *Fusarium* spp.

It becomes quite clear from the results, that whereas the percentages found on blotters decrease after water soaking, those obtained on the selective peptone-PCNB agar rise tremendously. It should be added here, that on the seeds soaked in terramycin a profuse growth of mycelium of *Fusarium* occurred, even to the extent that the seeds were hardly visible any more. On the seeds that had been soaked in water no mycelium could be seen at all, the fungus only growing out on the medium. This difference was felt to be the explanation for the fact that on blotters the percentage *Fusarium* obtained after water soaking always fell off.

Plating of soaked seeds containing *Septoria nodorum* on PDA also gave results quite different from those obtained in blotter tests (table 33). Agar testing of such seeds sometimes gives a false impression, due to increased interference by other fungi reducing the *Septoria* percentage found. If this is not the case, as in the above table, a reduction in the number of colonies with prolonged water soaking can be noted, those obtained becoming progressively smaller and slower growing.

TABLE 33. Effect of soaking in water and 200 p.p.m. terramycin solution for various periods of time at 20 °C on the percentage of *Septoria nodorum* subsequently found in blotter test and on PDA

soak-treatment	*S. nodorum* blotter test	potato dextrose agar (without NaClO pre-treatment)			
		S. nodorum	*Epicoccum*	*Alternaria*	*Mucor*
control	67	57	29½	31	few
24 hours in water	24	50*	37	32½	few
24 hours in terramycin	71	60	28½	16½	many
48 hours in water	3½	43½**	19½	20	few
48 hours in terramycin	76	56½	27	28	many
72 hours in water	0	37½***	23½	17	few
72 hours in terramycin	77½	all plates overgrown by *Mucor*			

*, ** and *** colonies progressively becoming smaller with increased length of soaking
*** colonies very tiny

Table 34 finally gives the results of an experiment with an oat seed sample containing *Helminthosporium avenae*. Much trouble was experienced with *Mucor* development. It will be noted from the table, that the total percentage of *H. avenae* found did not decrease. The morphology of the colonies obtained

TABLE 34. Effect of soaking of oat seed for various periods in water and 200 p.p.m. terramycin solution on the percentages of some fungi and the appearance of colonies of *Helminthosporium avenae* found in subsequent agar tests on PDA

soak-treatment	*Fusarium*	*Epicoccum*	*Alternaria*	*Helminthosporium avenae*					*H. sativum*	*Mucor*
				normal colonies with coremia	normal colonies with coremia and pycnidia	white flat colonies with pycnidia	White flat colonies without pycnidia	total		
non-presoaked control	6	58	7	60	–	1	–	61	4	many
24 hrs in water	6	33	7	37	21	22	4	84	3	very few
24 hrs in terramycin	19	54	7	61	–	–	–	61	6	many
48 hrs in water	10	1	5	61	1	1	6	69	4	few
48 hrs in terramycin	5	65	2	58	7	9	–	74	–	very many
72 hrs in water	3	3	3	26	–	6	49	81	1	few
72 hrs in terramycin	all plates overgrown by *Mucor*									

after soaking was, however, very different. Instead of the characteristic colonies with white tufts (coremia), a large proportion consisted of white flat colonies, many of which contained pycnidia. Many colonies had a sector which was still of the normal type with white tufts. When such colonies were transferred to fresh PDA medium they retained the abnormal morphology even after repeated transfers.

Apparently some factor of the soaking had induced the fungus to change its appearance. The pycnidial form is mentioned by MALONE & MUSKETT (1964) as being observed by them on malt extract agar. In the blotter test with soaked seeds pycnidia were also found, although to a lesser extent.

It was concluded, that in the long run soaking in water reduces the viability of the fungus present. After longer soaking periods the colonies become gradually smaller, or change their appearance.
One fungus is probably more sentitive than another, whereas the amount of inoculum present and the depth of infection are also important. This is illustrated by *Epicoccum* in table 34, which by NaClO pretreatment was reduced from 55 to 12% and was also strongly reduced by soaking.

3.19 THE INFLUENCE OF HYPOCHLORITE PRETREATMENT ON THE WBE AND THE SE

Hypochlorite pretreatment of seeds generally reduces their bacterial flora, as will be shown in section 3.20 (table 37). Its influence on the WBE and soak effect was therefore examined. As this pretreatment often reduces the total amount of infection obtained (section 5.3 and ch. 6), it is necessary to compare the results with those of similarly pretreated controls. In table 35 the results of 'wet' and 'dry' blotter tests for *Stemphylium radicinum* in carrot seed are given, with and without preliminary treatment with hypochlorite. There is still a WBE left after the pretreatment, but it is much smaller than for that of the non-pretreated objects. It was concluded that this agrees well enough with the fact, that superficial infection is more sensitive to the WBE. This would then be in agreement with the findings on the sensitivy of artificial inoculation as applied in section 3 4.

TABLE 35. Effect of pretreatment with ½ % NaClO for 10 minutes on the WBE for 5 carrot seed samples infected with *Stemphylium radicinum*

sample	non-pretreated		NaClO-pretreated	
	'wet' blotters	'dry' blotters	'wet' blotters	'dry' blotters
1	7	26½	12½	14¾
2	6½	9½	6¾	6
3	18½	29	15½	17
4	53¾	70¾	31¾	40½
5	17	30½	14	19½
average	20.6	33.2	16.0	19.6

In table 36 the results of blotter and agar tests are given for two wheat seed samples with *Fusarium* infection, soaked in water for 24 hours, with and without preliminary treatment with NaClO. The results clearly show that in blotter tests the SE after hypochlorite pretreatment is still present. This was explained by the fact, that the bacterial population built up quite rapidly again and could spread easily through the soaking water from seed to seed. On blotters this spread is probably less easy. Another factor that may have contributed to the observed differences came to light in later work (cf. section 3.18; ch. 5).

Hypochlorite after-treatment of soaked seeds caused a severe reduction of the *Fusarium* percentages found on both media, due probably to an easier penetration of the disinfectant into the soaked seeds.

TABLE 36. Effect of pretreatment with 1% NaClO solution for 10 minutes on the soak-effect for 2 samples of wheat seed infected with *Fusarium* spp

sample	non-presoaked control				soaked in water for 24 hours					
	without NaClO pretreatment		with NaClO pretreatment		without NaClO pretreatment		with NaClO pretreatment		without NaClO pretreatment, but with NaClO after-treatment	
	blotter	PDA	blotter	PDA	blotter	PDA	blotter	PDA	blotter	PDA
1	50¼	41	35½	42½	25¾	42	17¼	40½	11⅛	14
2	33½	21⅛	10¾	23½	20½	23	5¼	24½	3¼	15½

The conclusion drawn from this section may also be, that whereas hypochlorite pretreatment in many instances reduces the bacterial flora, it does not eliminate the WBE or the SE, although it may reduce them.

3.20 BACTERIAL ANTAGONISM IN THE AGAR METHOD

Bacterial antagonism in agar tests is more easily recognized as such than it usually is in blotter tests. In agar tests even with low pH media such as PDA or MA large numbers of bacterial colonies may sometimes grow from the seed. Especially if these bacteria are of the spreading type they often prevent fungi from growing. If fungi grow out, their development may be retarded and the colonies may become abnormal. When a hypha of a fungus colony comes into contact with these bacteria, the latter may spread rapidly along it and change the appearance of the colony. This can especially be observed with *Fusarium* spp. growing from wheat seed. The colonies turn brown and the aerial mycelium is flattened and adhering to the agar surface. This renders fungus identification much more difficult. The colonies usually continue growth at a slower rate after the bacteria reach and affect them. They remain small and are often reduced to merely a small brown spot. If transfers are made to fresh plates, they give rise to the same type of bacteria-contaminated, brown, restricted and abnormal colonies. It is necessary to take mycelium from the edge, as from more inward parts of the colony new growth is rarely obtained. Under the microscope the hyphae from such colonies are for the larger part necrotic.

For seed testing this bacterial antagonism is quite a nuisance. It is especially encountered in experiments with seeds that have not been pretreated with hypochlorite solution. If the seeds are pretreated, bacterial antagonism is much less important in the majority of cases although not in all. Especially with car-

rot and grass seed samples large percentages of the seeds sometimes give rise to abundant growth of bacteria, thereby decreasing the percentage of pathogen infection obtained. In order to get rid of this bacterial antagonism, 25 p.p.m. terramycin was added to the agar medium before pouring plates. This virtually eliminated bacterial growth on the agar. It was then possible to compare media with and without terramycin.

In table 37 the percentages of seeds with bacteria and a number of fungi are given, obtained on agar media with and without terramycin, as well as with and without pretreatment with NaClO. It can be seen that both terramycin and NaClO pretreatment reduce the percentage of seeds yielding bacterial growth, although the former is more efficient than the latter in this respect. It can also be seen that the percentages of fungi are somewhat increased by the addition of terramycin. The NaClO pretreatment, however, also decreases the percentage of fungi found with the exception of *Fusarium nivale*. The reason for this 'NaClO-effect' will be dealt with in ch. 5 and 6.

TABLE 37. Effect of NaClO pretreatment (½% for 10 minutes) and of the addition of 15 p.p.m. terramycin to PDA on the average percentages of some fungi and bacteria obtained from 10 wheat seed samples

NaClO pretreat-ment	terramycin	*F. nivale*	other *Fusa-rium* spp.	*Septoria nodorum*	*Epicoccum*	*Alternaria*	Bacteria
–	–	*16.1*	*12.1*	*14.1*	*33.6*	*38.9*	*54.4*
–	+	20.3	15.1	15.9	38.5	41.7	1.1
+	–	18.8	9.1	10.7	19.8	36.9	10.0
+	+	20.9	9.5	12.7	21.1	35.1	1.1

It follows that the fungus percentages are decreased by bacterial antagonism. The measure of the decrease can be expected to be a function of the quantitative and qualitative character of the bacterial flora of the samples. This was not examined any further, except for the observation that the number of bacterial colonies alone gave no good correlation with the decrease in percentage of fungal infection obtained (Fig. 3 and table 37). The species involved and probably also the growth conditions can be expected to play an important role. The latter factor is quite clear if we examine the difference between the effect of bacterial antagonism with different media, temperatures, etc. In table 38 the results of testing seeds on three media, with and without terramycin, are given as an illustration.

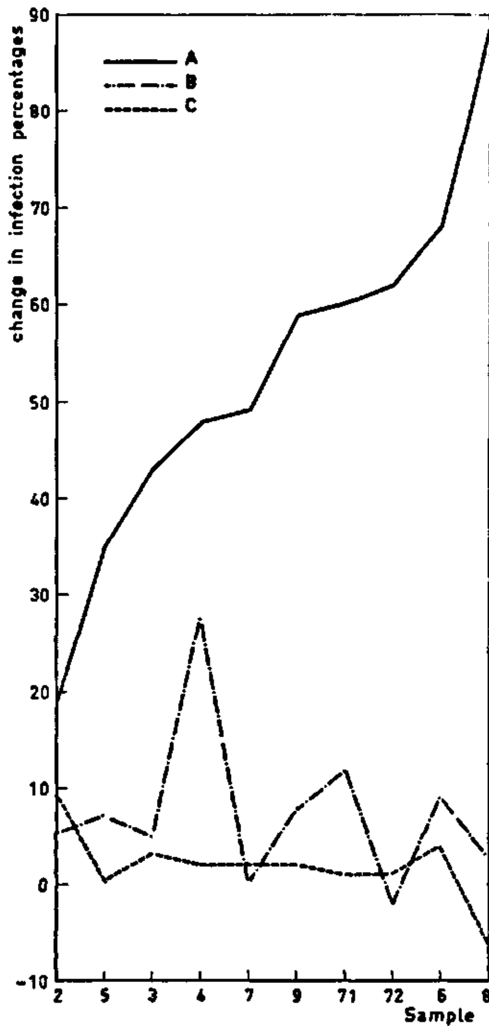


FIG. 3. Change in ratio of infection percentages found for bacteria, *Fusarium* spp. and *Septoria nodorum* brought about by 15 p.p.m. terramycin in the PDA medium.

A = percentage bacteria on PDA without terramycin minus percentage bacteria on PDA with terramycin

B = percentage *Fusarium* spp. on PDA with terramycin minus percentage *Fusarium* spp. on PDA without terramycin

C = percentage *S. nodorum* on PDA with terramycin minus percentage *S. nodorum* on PDA without terramycin

TABLE 38. Effect of the addition of terramycin to 3 agar media on the percentages obtained for some fungi in a wheat seed sample – no NaClO pretreatment used

terramycin	agar medium	*F. nivale*	*F. graminearum*	*F. avenaceum*	total *Fusarium*	*Septoria nodorum*	*Epicoccum*	*Alternaria*	Bacteria
0 p.p.m.	MA	33	7	5	45	6	23	26	52
15 p.p.m.	MA	34	7	5	46	4	26	27	0
0 p.p.m.	PDA	29	3	4	36	4	24	21	57
15 p.p.m.	PDA	31	6	6	43	5	23	25	0
0 p.p.m.	NA	24	5	2	31	3	17	19	63
15 p.p.m.	NA	31	7	9	47	4	23	27	2

3.21 The moisture effect in silversand and soil

In order to examine the effect of soil moisture on the development of a seed-borne infection, some experiments were carried out with wheat seed infected by *F. nivale*. In the experiments in which silversand was used, the moisture content was adjusted by adding one volume of water to 5 or 6 volumes of air-dry silversand, resulting respectively in 'wet' or 'dry' silversand. The soil used in the experiments was judged as being on the 'wet' or on the 'dry' side by sight and touch.

Experiments were carried out with a wheat seed sample artificially infected with *F. nivale* and one with a high percentage of natural infection. The sample used for the artificial infection was free from seed-borne *Fusarium* spp. and other pathogens. It also contained but very few fungi in general. Artificial inoculation was carried out by mixing 2½ ml of a spore suspension, containing about ½ million spores per ml, with 500 seeds. Such inoculations were made with seeds which had either been pretreated or not with 1% NaClO for 10 minutes.

The results are given in table 39. It is quite clear that pretreatment of the seed considerably increases the effect of the inoculation. As the seed lot used contained hardly any seed-borne fungi, the effect must be due to the bacterial seed flora. On Difco Bacto Nutrient Agar colonies were obtained from 67% of the seeds. The effect was somewhat greater in the 'wet' object than in the 'dry' one. These results were in agreement with those of previous sections.

Table 41 gives the results of an experiment with a wheat seed sample naturally infected with 59½% *F. nivale* and not too much other fungi (Sample A of table 40). Here also the 'wet' objects gave less disease development than the 'dry' ones, except the object with non-sterile clay at 15°C. That *F. nivale* caused

TABLE 39. Effect of artificial inoculation with *F. nivale* of either NaClO-pretreated (1% for 10 minutes) or non-pretreated wheat seeds on the percentages of total emergence and of diseased plants after sowing in relatively 'dry' or 'wet' silversand in 'tents' kept at 7 °C

	NaClO pre-treatment	'dry' silversand		'wet' silversand	
		%emergence	% diseased plants	%emergence	% diseased plants
not inoculated	–	85½	½	88	½
not inoculated	+	87	0	89½	2
inoculated	–	79½	25½	85½	14
inoculated	+	51½	41	56½	40½

TABLE 40. Percentages of some fungi found in the wheat seed samples, A and B (cf. text), on potato dextrose agar after NaClO pretreatment (1% for 10 minutes)

method	fungi	sample A (Gaby '66)	sample B (Tadorna '66)
agar	*Fusarium nivale*	59½	11½
agar	*F. graminearum*	1	2
agar	*F. avenaceum*	5	7
agar	total *Fusarium*	65½	20½
agar	*Septoria nodorum*	0	62½
agar	*Epiccocum*	12½	15
agar	*Alternaria*	23	8½
blotter	*Fusarium*	77	45½
blotter	*Septoria nodorum*	0	87½

TABLE 41. Effect of relative 'wetness' of silversand and sterile or non-sterile clay loam on the percentages of total emergence and of diseased plants obtained after sowing wheat seed (of sample A of table 40) in these media in 'tents', after 30 days at 7 °C or after 18 days at 15 °C

temperature	medium	'dry' medium		'wet' medium	
		% emergence	% diseased seedlings	% emergence	% diseased seedlings
7 °C	silversand	45½	28½	49	23½
	sterile clay loam	43½	30	51	17
	non-sterile clay loam	54½	20½	61½	14½
15 °C	silversand	64	51	68½	37
	sterile clay loam	69½	41	76½	18½
	non-sterile clay loam	76	1	76½	0

some disease at 15 °C in silversand and sterile soil, was probably due to the high infection level and the low numbers of other fungi in this special sample. Generally it is very hard to obtain disease symptoms of *F. nivale* at this temperature, most likely because of antagonism by other members of the seed microflora and of the soil microflora in non-sterile soil.

The results in sand and soil are an indication that here the same mechanisms play a role as on blotters. The situation is, however, more complicated due to the presence of soil-borne bacteria in non-sterile soil.

3.22 The effect of soaking, bacterization and other pretreatments of seeds on disease development in silversand and soil

The most frequently observed species of *Fusarium* in wheat seed samples at the Govt. Seed Testing Station at Wageningen during the course of the work here described was *F. nivale*. It had been found that this species usually only causes damage only in soil if the temperature was below 15 °C. For that reason the influence of bacterial antagonism in soil was studied at low temperature. Experiments with this infection were therefore carried out in climate rooms in so-called 'tents' (ch. 2), in order to keep the soil moisture constant. Table 42 gives the results of a number of pretreatments on the performance of wheat seeds (sample A of table 40) in silversand and silty clay loam at low temperatures. Bacterization was carried out with *Bacillus cereus* var. *mycoides* (section 3.15) by mixing a water suspension, made from cultures on nutrient agar, with the seeds. The influence of soaking and bacterization is quite evident.

With another wheat seed sample, which carried a heavy load of *Septoria nodorum* (B of table 40), a similar experiment was carried out in the greenhouse, kept at 15 °C. The seeds were sown at a depth of 1½–2 cm in 4 rows of 50 seeds in greenhouse benches filled with silty clay loam. The distance between the rows was 10 cm and the repetition of the rows of the same object was randomized. After 3 weeks the seedlings were inspected for *Septoria* symptoms (large brown coleoptyle lesions). The results are given in table 43. They show the same trend as those of table 42.

As to the effect of terramycin in soil it can be noted, that it results in a remarkable depression of emergence and increase of abnormal seedlings. In how far this is due to increased fungus growth, and in how far to some direct effect of the antibiotic on the seedling is hard to tell. Samples with low infection percentages often also give a retarded development and an increased number

TABLE 42. Effect of various pretreatments of wheat seeds (sample A of table 40) on percentages of total emergence and of diseased plants after sowing in different media in 'tents' kept for 30 days at 7°C or for 18 days at 12°C

temperature	pretreatment*	silversand		sterile clay loam		non-sterile clay loam	
		% emergence	% diseased seedlings	% emergence	% diseased seedlings	% emergence	% diseased seedlings
7°C	none	55½	26½	54	13½	80½	21½
	NaClO	39	12	39	11½	66½	52½
	terramycin	81	26½	77½	5½	94	13
	broth	74	30½	79½	15	97	12
	Bac.	78½	22½	80½	7	87	30½
	cer.	95	1½	93½	2½	94	0
12°C	none	76½	25	77	19	87	21
	NaClO	15½	9½	16	10	26	13
	terramycin	90	8	90½	9½	97	25
	broth	89	10	93	13	89	20½
	Bac.	93	14	97	12	96½	8½
	cer.	93½	0	96½	½	97	0

* NaClO = pretreated with 1% NaClO for 10 minutes; terramycin = soaked for 1 hour in 200 p.p.m. terramycin solution; broth = soaked for 1 hour in Difco nutrient broth; Bac. = inoculated with watery suspension of *Bacillus cereus* v. *mycoides*; cer. = shaken with ceresan dust (Bayer)

TABLE 43. Effect of various pretreatments of wheat seeds (sample B of table 40) on percentages of total emergence and of diseased plants obtained 3 weeks after sowing in benches with silty clay loam in the greenhouse kept at 15°C

pretreatment	emergence	diseased seedlings
control	91	83
shaken with ceresan	99½	4½
1% NaClO for 10 minutes	97	16
water for 24 hours	92½	80½
water for 48 hours	91	40½
nutrient broth for 24 hours	98½	78
nutrient broth for 48 hours	96½	35
100 p.p.m. terramycin tor 24 hours	83½	80½
200 p.p.m. terramycin for 24 hours	72½	67½
200 p.p.m. terramycin for 48 hours	65	54
water for 24 hours, followed by 1% NaClO for 5 minutes	99	15
water for 24 hours, followed by 200 p.p.m. terramycin for 1 hour	84	80
1% NaClO for 5 minutes, followed by water for 24 hours	95	14½
1% NaClO for 10 min., followed by water for 24 hours	98	26½
1% NaClO for 10 min., followed by water for 24 hours, and then by 200 p.p.m. terramycin for 1 hours	91	48½

of abnormal seedlings. This is especially the case with older samples and perhaps the elimination of antagonistic bacteria enables saprophytic and storage fungi to grow so well as to lower the seed vigour (cf. section 8.1). In fresh samples with low infection percentages the effect was less severe. Streptomycin and chloramphenicol had the same effects as terramycin in this connection.

Other factors influencing the infection percentage obtained in soil will be discussed in later chapters.

3.23 DISCUSSION

Bacteria and fungi prefer different conditions for growth and development. It is well-known, for example, that most bacterial species prefer higher pH levels than fungi. In studying the WBE and the related SE, it was found that high substrate moisture stimulates the bacteria more than the fungi. Also a higher temperature, as a rule, favours the bacteria.

So, by conditions promoting their growth, the bacteria will get the lead over the fungi and they will thereby prevent a large proportion of the fungi from growing. This proportion apparently depends on the relative lead that the bacteria are gaining. This appears from the fact that the WBE and the SE can be made more severe by increased wetness of the substrate, or length of soaking, by higher pH, and temperature, by addition of nutrient broth, etc. It is probably also influenced by properties of the fungal population, notably the quantity of fungal inoculum being present and its depth of penetration (cf. ch. 6). In general we may expect that the latter will increase with the former, so that we may perhaps replace it by the term 'severity' of the infection which would cover both. If the infection is more severe, it is apparently less easily influenced by bacterial antagonism. Superficial infection is very sensitive, as follows from the results of experiments with artificially inoculated seeds (section 3.4).

After water soaking of seeds showing no WBE the percentages of infection found on blotters were usually decreased, on agar with terramycin not. This was especially evident for *Fusarium* spp. on a selective medium. Apparently there is even an initial period of stimulation (positive soak effect) followed by fungistasis caused by bacteria. If this fungistasis lasts too long, the fungi will gradually start to decline. So severe infections can be expected to remain unaffected for a longer time than slight ones. The kind of fungus involved may also be important. All the experiments reported in this chapter lead to the conviction, that WBE and SE as exhibited in blotter tests and in soil are due to bacterial antagonism. The observations made show that for such infections as

B. cinerea in flax seed and *Fusarium* spp. in wheat seed, soil moisture may be rather important. In case of the experiments with flax seed in petri-dishes with silversand it appeared that too dry conditions can also hamper the fungus. This corresponds with the observations of VAN DER SPEK (1965) on the influence of dry soil surface. Moisture is necessary for the fungus, but too much stimulates bacteria more than the fungi.

NEWHOOK (1951) mentions the decreased number of lettuce plants attacked by *B. cinerea* on wet spots in the field. In the case of *Fusarium* infections of cereals COLHOUN & PARK (1964) reported on the preference of *Fusarium* for dryer soil conditions in field experiments with artificially inoculated seeds. MUSKETT (1937) reported the same preference in case of *Helminthosporium avenae*. The stimulation of rapid bacterial development by moisture is among others reported by VERONA (1963), who states that it suffices to put a drop of sterile water with some seeds on a microscope slide to induce an abundant bacterial development after only a short period of incubation. He gives photographs of such preparations from seeds of wheat, flax, cabbage, clover and alfalfa.

If seeds showing WBE are put on 'wet' blotters, the difference in speed of moisture uptake of the seed, as compared with that on 'dry' blotters, is apparently such that it causes a relative speeding up of bacterial development giving the bacteria a lead over the fungi. The infections first to be affected are probably the less severe ones. If antibiotics are added either to the blotters, or to the soaking water in case of seeds showing the SE, bacterial growth is checked and the fungi are given the undisputed lead. In case the blotters are kept on the 'dry' side, or when the pH is kept low, the fungi are also given the lead over the bacteria, but not an absolute one as in the case of antibiotics. If one of both groups has taken the lead, the other is at a disadvantage although it may perhaps still develop some activity, as is for instance the case when bacterial colonies on agar are growing out belatedly into fungal colonies, thereby changing the appearance of the latter as well as their rate of growth and often inducing necrosis or sometimes lysis of hyphae. If the bacteria have the lead, fungi may appear somewhat retarded, growing out even over bacterial colonies after these have apparently stopped rapid growth. Usually, however, fungi do not grow out under such conditions, hence the lower percentages obtained on agar without terramycin (table 14).

It could also be observed, that wheat seeds after having been soaked in water, yielded just as many colonies as without soaking when plated out on agar with terramycin, but the surface of the seed itself remained free from fungi. On seeds

soaked in terramycin, on the other hand, the whole seed was covered by fungal mycelium as well. On the water-soaked seeds the bacteria retained the lead, as the terramycin in the agar hardly reached them.

The difference in results of inoculation with *F. nivale* on seeds with and without NaClO pretreatment (section 3.21) reminds one of similar observations by SIMMONDS (1947) for *Helminthosporium sativum*. This author showed that incubation of wheat seeds on blotters in a moist chamber increased the degree of antibiosis against artificial infection by *H. sativum*. This effect could be abolished by treatment of the incubated seeds with formalin before inoculation with *H. sativum*. LEDINGHAM *et al.* (1949) even stated that differences in susceptibility of two Canadian wheat varieties to *H. sativum* might be due to differences between the microfloras of their seeds.

The results obtained with the soaking of seeds in water recall of course the use of simple water soak methods for control purposes. This possibility was first demonstrated by TYNER (1953, 1957) for the control of loose smut of barley and wheat and subsequently reported on by other workers for various seed-borne diseases. Several explanations have been offered for its mode of action, which are mentioned by IVANOV (1958) and reviewed by WALLEN (1964). Inhibition by microbial products, toxic agents in the steepwater (form the seed), depletion of oxygen, nutrient impoverishment of the seed, and a combination of these factors have been supposed. WALLEN (l.c.) states that: 'unfortunately no theory has been verified, because of the fact that much of the work has been devoted to practical reasons of control with fewer studies aimed at determining the principles for such control'.

In the SE here described anaerobiosis also might play a role. It is perhaps existing around the seed on 'wet' blotters showing WBE. A moisture film is formed around the seeds, either by capillary action because of their small size, or by the presence of a mucilaginous seed coat layer (flax and to a lesser extent cabbage). In the present experiments bacterial activity apparently is the main factor.

It was not our intention to study the background of the SE, but only to study the importance of bacterial antagonism for seed health testing. Hence no special experiments were made in this direction. The experiments on control of seed-borne infections by TYNER and others were mostly carried out at somewhat higher temperatures and for longer periods of time. Hence secondary factors such as the formation of organic acids may become reltively more important. In our experiments butyric acid could often be smelled.
ARNY & LEBEN (1954) reported very good control of *Helminthosporium sativum*

and *H. victoriae* in barley and oat seed, respectively, after soaking for 56 hours at 24–26 °C. They state, however, that the presence of the antibacterial antibiotics aureomycin, streptomycin sulfate or terramycin in the soak water did not lessen control. They do not give the concentrations used. This is thus at variance with our own observations. We invariably found that the addition of sufficient terramycin to the soaking water decreased or nullified the SE. The same applies to the addition of terramycin to blotter tests with infections showing WBE.

It was observed that terramycin and also streptomycin and chloramphenicol when present in the soaking water had a retarding influence on subsequent development of the seedlings on blotters as well as in soil. In how far this was due to a direct effect of the antibiotic on the seedling or to an increased fungal development is hard to tell. The first factor cannot be ruled out entirely so that in that case the factor host resistance as discussed in the next chapter would be involved as well. An interesting point in this connection is the observation made by WAGGONER (1956), who found that streptomycin significantly increased the growth of *F. sambucinum* on potato slices and had no effect in vitro. VÖROS et al. (1957) reported, however, a streptomycin-induced resistance of potato against *Phytophthora infestans* acting via stimulation of polyphenolase production.

As to the practical consequences of the findings reported in this chapter, it can be mentioned that in the first place our insight into the factors influencing the results of incubation tests for seed health examination has been enlarged. Besides physical factors such as temperature, humidity, substrate moisture content, light, etc., we also have to do with biotic factors. The biotic factors are influenced by the physical factors. Hence the influence of physical factors on the outcome of seed health test results may sometimes be an indirect one, as we have seen for instance very clearly in this chapter for the factor substrate moisture.

The biotic factor of bacterial antagonism can be supposed to vary from seed to seed, and also from seed lot to seed lot. We can expect fluctuations between the results of seed health tests between stations or even between tests at the same station, if the incubation conditions are not the same and constant. The WBE was dicovered as a result of looking into the cause of such fluctuations. Apparently the quantity of substrate moisture is an important factor and this factor is hard to standardize. It is, however, simple to eliminate bacterial antagonism entirely by the use of antibacterial substances and thus to eliminate an important cause of fluctuation.

It can be objected that in this way less severe infections of individual seeds

will also be counted, whereas these will have little chance of producing disease in the field as under agricultural conditions antagonism will wipe them out. To this subject we will return in ch. 6. Stimulation of the seed's own bacterial flora or extra bacterization of seeds with seed-borne bacteria, strongly decreases disease development. Bacterization of seeds was already used for controlling disease in prewar experiments by Russian workers (NOVOGRUDSKII *et al.*, 1937; BERESOVA, 1939), who used species of soil-inhabiting bacteria.

The results reported in this chapter may also pertain to the testing of seeds that show no WBE but do show a SE, in case they are tested in pure sand or in soil. Germination tests are sometimes carried out in sand, for example for cereal seeds. The seedlings severely attacked by *Fusarium* or *Septoria* are regarded as abnormal in this test. The percentage of discarded abnormal seedlings can be influenced by the moisture level of the sand.

4 HOST VIGOUR

4.1 INTRODUCTION

When working on the WBE a new phenomenon was met which led to the study of another factor influencing the results of incubation tests. It was the enormously increased percentage of *Phoma valerianellae* found in 2,4-D blotter tests as compared with normal ones. It was given the name of 2,4-D effect. Its study led to the realization of the importance of the factor 'host vigour'. A new testing method was then developed, viz. the freezing method.

4.2 THE USE OF 2,4-D IN SEED HEALTH TESTING

The sodium-salt of 2,4-D (2,4 dichlorophenoxyacetic acid) is sometimes applied in certain seed health tests in order to inhibit seed germination. It was first used for this purpose by HAGBORG *et al.* (1950) in the examination of bean seeds on agar for the presence of *Colletotrichum lindemuthianum*. They recommended 50 p.p.m. 2,4-D in the agar. Without it seedling growth soon makes the tests useless.

NEERGAARD (1956) added a 0.2% solution of 2,4-D to the blotters in testing for cabbage infections. In the Wageningen Seed Testing Station this 2,4-D blotter method has also been used for the routine examination of cabbage seeds *Phoma lingam* (DE TEMPE, 1961a). The 2,4-D blotter method has been included in the chapter on seed pathology of the 1966 ISTA Rules for testing of *Brassica* seeds. (ANONYMOUS, 1966).

The 2,4-D blotter method is also very useful for carrot seeds with *Stemphylium radicinum* and *Alternaria dauci*. In the ordinary blotter tests the inspection for the above pathogens is rather awkward due to the displacement of the seed coats on which the fungal structures are mainly found. Both methods give the same percentages, but the 2,4-D method takes far less time and the chance of error due to the human factor is much smaller.

NEERGAARD & SAAD (1962) and NOBLE (1965) reported an increased percentage of *Pyricularia oryzae* on rice seed in 2,4-D blotter tests as compared with normal blotter tests.

4.3 THE 2,4-D EFFECT

In chapter 3 we already met with the *Phoma valerianellae* infection of corn-salad seeds. This infection had some interesting aspects: it did not show any WBE but gave a positive soak effect, whereas *Botrytis cinerea* in the same seed disappeared completely after soaking. In section 3.4 we also saw that isolated true seeds gave a WBE in contrast to the complete 'seeds' (actually fruits).

Another interesting phenomenon was found. The *Phoma valerianellae* infection in the blotter test is detected by the presence of *Phoma* pycnidia on the seedlings. In 2,4-D blotter tests germination is not completely prevented for many kinds of seeds, but usually development of the seedlings is halted soon after germination: they remain small and are stunted and swollen. In the case of corn-salad this commencing germination is necessary for the observation of pycnidia on the rootlets. With the 2,4-D blotter tests for the cabbage and carrot

TABLE 44. The effect of the addition of 2,4-D to the blotters on the percentage of *Phoma valerianellae* in 6 corn-salad seed samples after 7 and 10 days of incubation

Sample	After 7 days		After 10 days	
	normal blotter test	2,4-D blotter test	normal blotter test	2,4-D blotter test
1	2	22½	7¾	38¼
2	¾	15½	5¼	35¾
3	1½	20	8	38¼
4	¾	16	5	27½
5	1¼	6¾	2½	16¾
6	1½	5½	3	24½
average	1.3	14.4	5.3	30.2

infections mentioned in section 4.2, germination is not necessary as here sporulation takes place on the seed surface itself.

In 2,4-D blotter tests with corn-salad the percentage of 'seeds' found with *Phoma valerianellae* was several times higher than in the normal test (table 44). This phenomenon was called the 2,4-D effect. It was later on found for some other infections as well. The observation of NEERGAARD & SAAD and of NOBLE with *Pyricularia oryzae*, as mentioned in section 4.2, was perhaps also due to this effect.

4.4 INFLUENCE OF 2,4-D ON FUNGI

There have been a number of reports on the effect of 2,4-D on fungi. In general it can be stated that the effect of 2,4-D varies with the formulation and concentration, the butyl ester of 2,4-D being more fungitoxic than its sodium salt and higher concentrations being proportionately more harmful (BEVER, 1948).

The same was observed in own experiments. Growth measurements were made at daily intervals for a number of pure cultures of fungi on PDA with various dosages of 2,4-D, the sodium salt ordinarily used in 2,4-D testing being used. Fig. 4 gives an idea of the results.

They do not show the complete picture as 2,4-D also induces abnormalities in the form of the spores. These are often irregularly swollen and misshapen. At higher concentrations the number of spores produced also tends to decrease. Table 45 gives the influence of 2,4-D on the percentage of wheat seeds giving rise to fungal colonies on PDA. Higher 2,4-D levels tend to decrease the percentages found. It also shows that the effect on the various fungi is not the same.

As to the effect of fungal development in host tissue there have been reports of inhibitory (GUISCAFRE-ARRILLAGA, 1949), as well as stimulatory effect (FEN-

TABLE 45. The effect of 2,4-D concentration in PDA on the average percentage of some fungi found in 10 wheat seed samples

p.p.m. 2,4-D	*F. nivale*	*F. graminearum*	*F. avenaceum*	*Fusarium* spp.	total *Fusarium*	*Septoria nodorum*	*Epicoccum*	*Alternaria*
0	9.6	4.4	2.5	0.7	17.2	9.3	22.5	37.2
25	10.7	4.6	1.9	0.3	17.5	8.6	24.7	39.0
50	8.3	3.9	1.9	0.4	14.5	11.5	21.4	39.6
100	5.9	4.2	2.2	0.6	12.9	10.1	24.3	40.6
200	4.3	2.1	1.6	0.4	8.4	7.4	16.3	36.7
500	practically no growth							

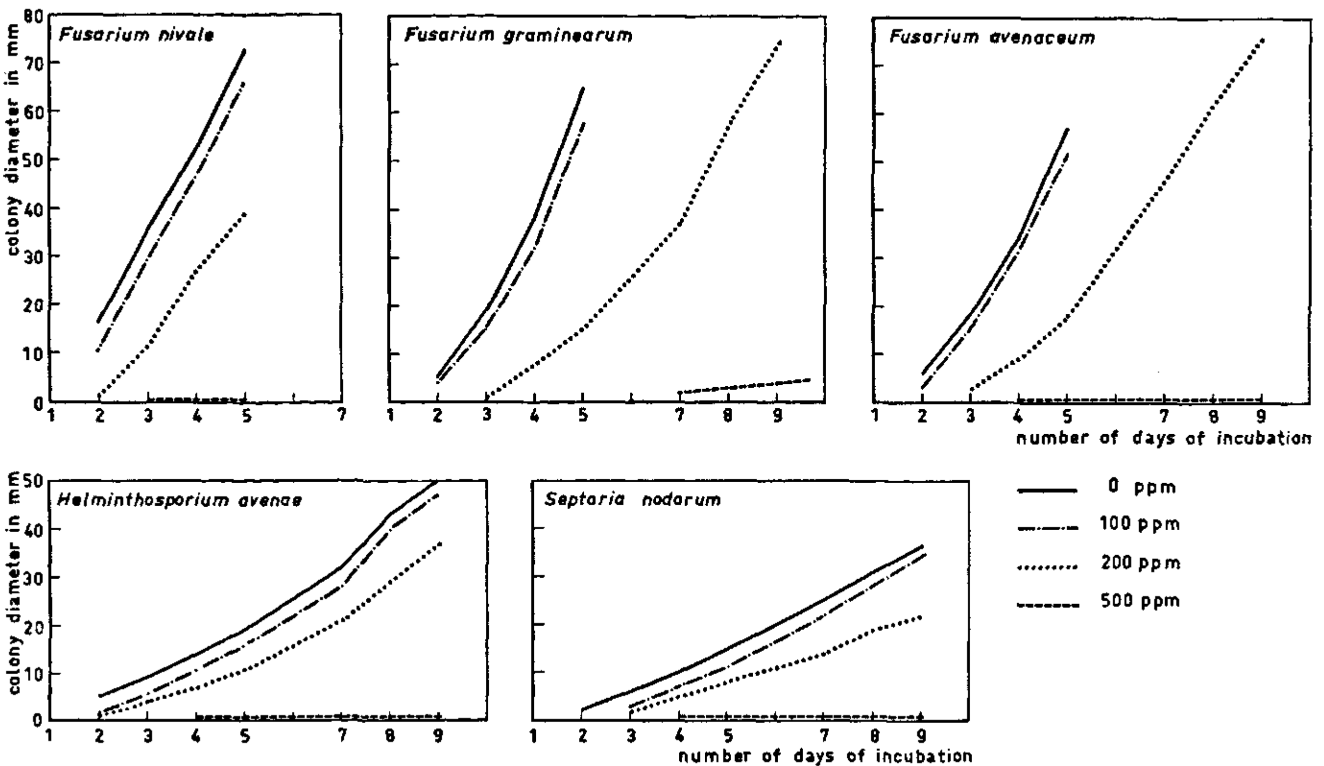


Fig. 4. Effect of different concentrations of 2,4-D in PDA on the growth-rate of pure cultures of some fungi (Average of 10 colonies per object)

NER & FATE, 1947; HSIA & CHRISTENSEN, 1951). The latter report describes an increased attack of wheat seedlings by *Helminthosporium sativum* after 2,4-D treatment.

The wheat seedlings were reported as being definitely weakened, hypertrophied and stunted, and when inoculated they became more heavily infected.

SIMMONDS (1963), reported increased susceptibility of banana fruits to *Colletotrichum* spp. after 2,4-D treatment. Apparently 2,4-D alters the host metabolism, making the plant either more resistant or more susceptible to fungal attack.

BUTLER (1961) states that seed damage, 2,4-D, insecticides, fungicides, and winter injury increase the susceptibility of wheat to *Fusarium*.

4.5 THE MECHANISM INVOLVED IN THE 2,4-D EFFECT FOR THE PHOMA VALERIANELLAE INFECTION OF CORN-SALAD SEED

For the study of the 2,4-D effect four large corn-salad samples were obtained. As pointed out in section 3.9, *Phoma*-infected seeds gave a positive soak effect. Soaked seeds of corn-salad gave a poorer germination and less vigorous looking seedlings. As 2,4-D causes something similar it was supposed that host vigour is involved. It was also observed that the longer the blotter tests were kept, the higher the *Phoma* percentage (cf. table 44). This also might be a matter of decreasing vigour. It could not be due to spread of the fungus from infected to non-infected seedlings, as the experiments were carried out with only 25 seeds per blotter.

First the effect of diminishing the host vigour by other means such as wilting, cutting of root tips and freezing was studied. These maltreatments were carried out after four days preliminary incubation at 20 °C, when the seedlings were about ½ cm long. This was the stage best suited for out purpose. Seedling development is desirable as the *Phoma* pycnidia, by which the fungus can be identified, are more easily formed and recognized on the seedlings than on the 'seeds'. Waiting any longer permitted complicating factors to play a role, such as a stronger development of saprophytic fungi. It can be seen in table 46 that the experiments supported the vigour hypothesis.

It should be added here that the infection percentage obtained for frozen seedlings from fruits (table 46) and from isolated seeds (table 47) is practically the same, but in the case of the seeds the percentage can still be increased by the addition of terramycin to the blotters. This latter must be entirely due to the elimination of bacterial antagonism, as in the killed seedlings host vigour

TABLE 46. The effect of various treatments of corn-salad 'seed' or seedlings on the per centages found for *Phoma valerianellae* in some samples

sample	control	2,4-D	mutilation*	freezing**
1	4	32	29	45
2	9	28	27	50
3	10	39	47	61
4	17	38	26	56
average	10	34	32	53

* cutting of root tips

** trays with pregerminated (4 days old) seedlings placed into a deep-freezer of about -20 °C overnight, after which incubation was continued

TABLE 47. Effect of removal of fruit structures and various modifications of the blotter test on percentages of *Phoma valerianellae* found in some corn-salad seed samples

sample	'wet' blotters	'dry' blotters	'wet' blotters with terramycin	'dry' blotters with		
				0.2% 2,4-D	freezing	freezing and terramycin
1	2	3	4	33	46	60
2	4	8	7	42	61	75
3	4	12	15	50	60	82
4	10	17	19	46	57	76
average	5	10	11	43	56	73

cannot be further reduced. That terramycin only increased the percentage on isolated seeds can be due to two reasons. The first is that isolated seeds show a WBE and the fruits do not. The former are flat and in direct contact with the blotter and so more easily influenced by the terramycin than the round and larger corky fruits. The second reason may be the greater importance of interfungal antagonism in the fruits.

For verifying the host vigour hypothesis the samples were also tested by the agar method, in which host vigour does not play a role (table 48). In table 49 the results of various blotter and agar tests, taken from previous tables, have been placed beside each other. It can be clearly seen that the percentages obtained on agar are high and those on ordinary blotters low. Freezing of fruits increases the blotter test results to a higher level than those found on agar. If free seeds are plated on agar, however, the figures obtained for *Phoma valeria-*

TABLE 48. Effect of removal of fruit structures and NaClO pretreatment on agar test results for some corn-salad seed samples (7 days at 20 °C on PDA)

sample	structure tested	NaClO pre-tretment	infection percentages found for		
			Phoma valerianellae	*Botrytis cinerea*	*Fusarium* spp.
1	whole fruits	–	23	2	9
		+	29	0	5
	free seeds	–	66	5	0
		+	10	0	0
	seedless fruits	–	27	1	13
		+	23	0	10
2	whole fruits	–	34	6	21
		+	44	8	15
	free seeds	–	80	16	14
		+	40	1	3
	seedless fruits	–	lost by *Mucor*		
		+			
3	whole fruits	–	46	3	13
		+	35	1	10
	free seeds	–	78	6	2
		+	19	0	0
	seedless fruits	–	42	3	14
		+	32	1	9
4	whole fruits	–	58	1	27
		+	55	0	22
	free seeds	–	77	6	11
		+	34	0	3
	seedless fruits	–	57	0	20
		+	42	0	16
average	whole fruits	–	40	3	18½
		+	41	2	14
	free seeds	–	75	8	3½
		+	26	4	1½
	seedless fruits*	–	40	1	6
		+	32½	½	9½

* calculated by extra-polation for second sample

TABLE 49. Comparison of the percentages found by various blotter and agar tests for *Phoma valerianellae* in corn-salad seeds

sample	blotter tests					agar tests	
	normal	freezing	normal	freezing	freezing with terramycin	with NaClO	without NaClO + terramycin
	fruits	fruits	seeds	seeds	seeds	fruits	seeds
1	4	45	5	46	60	29	66
2	9	50	8	61	75	44	80
3	10	61	13	60	82	35	87
4	17	56	20	57	76	55	77
average	10	53	11½	56	73¼	40¾	75¼

nellae and *Botrytis cinerea* can be explained by the elimination of antagonism due to the fruitborne micro-flora. *Fusarium* spp. are largely removed.

Hypochlorite pretreatment scarcely harms the infections in the fruits, which are probably too deep-seated, but it appreciably reduces the infections in the free seeds which are apparently rather superficial. It appears that using isolated seeds without NaClO pretreatment in the agar method is necessary for obtaining the same infection percentages as in the blotter method with freezing.

4.6 THE FACTOR HOST VIGOUR FOR PHOMA VALERIANELLAE INFECTION OF CORN-SALAD SEED IN SOIL

Some tests were carried out with corn-salad seeds in silversand and soil. In such tests the symptoms caused by *Phoma valerianellae* were damping-off and red stripes on the stems; there was also pre-emergence death of plants. The disease percentage found amounted to roughly half the infection percentage in the blotter tests with frozen 'seeds'. It was thus much higher than the percentage obtained in ordinary blotter tests.

An interesting observation was made on seedlings grown in silversand which seemed quite healthy. When they were dug out and the roots cleaned with water, quite a number of healthy-looking seedlings were seen to have just one or a few brown lateral rootlets. When these were inspected with a stereoscopic microscope *Phoma* pycnidia were found, and the fungus could be isolated. Afterwards such roots were also observed on plants grown in ordinary soil. If the number of such plants with *Phoma* on some rotting roots was added to the

disease percentage noted directly, the figures came much closer to those for blotter tests with lowered host vigour.

DE JONG (1967) observed, that when apparently healthy corn-salad seedlings were transplanted, an outbreak of the disease occurred so that many additional plants died. This was also observed by the present author. Apparently any factor which decreases host vigour – such as transplanting – lowers the resistence of the host, thus improving the possibilities for the fungus to cause disease. In the case of this particular infection seed transmission is probably the main source of inoculum. The pathogen causes a certain amount of deaths after transplanting, perhaps also spreading the disease to adjacent plants. The rest of the infection apparently continues a more or less latent existence, living on dead or nearly dead host tissue. When the mature plants are dying off the fungus probably succeeds in invading the fruits tissues and thus the cycle will be closed.

The cycle described probably holds true for some other seed-borne *Phoma* infections as well, for instance *Phoma betae* in beet seed and *Phoma lingam* in cabbage seed. In the latter case transplanting from the seed bed to the field may cause infections that appear gradually and may even remain dormant until the cabbage head is harvested and stored (VAN BRAKEL & DE KRAKER, 1963).

4.7 HOST VIGOUR AND PHOMA BETAE INFECTION OF BEET SEED

As pointed out in section 4.6 the *Phoma* infections of corn-salad, beet and cabbage have many points in common. For that reason the influence of host vigour on testing for *Phoma betae* was also examined. The blotter test used was the routine method of the Wageningen Station, viz. 12 days in the dark at an alternating temperature of 20°–30°C. The inspection after incubation is difficult. In routine work this leads to overlooking of infections and aberrant results, especially in the case of poorly germinating or very dirty samples. With beet seeds the 2,4-D and freezing effects are less easily observed due to the development of saprophytes. Similar as with corn-salad seed a certain amount of germination is necessary, as the pycnidia are hard to find on the seed clusters. In spite of the large error it was quite clear that diminished host vigour increases the percentage of *Phoma betae* found, as can be seen in table 50. Here also the results of agar tests for the same sample are given, which are higher than those of the 2,4-D or freezing methods.

Later it was found that in blotter tests at a constant temperature of 20°C under NUV light, pycnidia are more easily obtained on the clusters themselves. This results in infection percentages comparable to those on agar (table 51).

TABLE 50. Effect of various modifications of blotter and agar tests on the average percentage of *Phoma betae* found in 10 beet seed samples

normal blotter test	2,4-D blotter test	freezing method	freezing method + terramycin	PDA	
				without NaClO	with NaClO
24.7	38.8	44.0	48.1	57.8	56.3

TABLE 51. Effect of NUV light and NaClO pretreatment on the average percentages of *Phoma betae* found in blotter tests with 7 beet seed samples. Agar test results are given for comparison (7 days at 20°C on PDA)

testing conditions	pycnidia on:	*Phoma betae*
normal blotter test		
(12 days 20/30° in darkness)	seedlings	35.9
	clusters	7.1
	total	43.0
blotter test		
(7 days at 20°C + NUV light)	seedlings	0.6
	clusters	79.1
	total	79.7
agar test on PDA		
(after NaClO pretreatment)	*Phoma* colonies	74.1

As the cluster tissue can be regarded as non-living, this was another argument in favour of the host vigour hypothesis. It also follows from table 51 that in the old blotter test carried out in darkness conditions are sub-optimal for fructification of the fungus. In blotter tests with NUV light they were quite favourable for pycnidia formation. It thus follows that the results of the old blotter method could be improved by:

a. reducing host-vigour, e.g. by 2,4-D or freezing, or by
b. promoting fructification on the clusters themselves by NUV light.

The agar method for beet seed does not only give much higher percentages than the old blotter test, but it is also easier and more pleasant. Pretreatment with NaClO in agar tests considerably reduces growth of saprophytic fungi (notably *Mucor*), without appreciably reducing the *Phoma* percentage found. Inspection of the plates is easy and quickly done. The best incubation regime found was 4 or 5 days at 20°C in the dark followed by 2 days at 20°C in NUV light. The NUV period is not strictly necessary but it stimulates the fructifica-

tion of the fungus. The colonies are greenish and have a characteristic radiating structure. If the dishes are kept against the light the pycnidia are easily observed. They often produce a cream-colored spore slime. On malt extract agar (Oxoid) colony appearance is somewhat less characteristic. There is more aerial mycelium confusing the radiating pattern and there are less pycnidia, which are more difficult to observe.

The agar method has since been adopted at the Wageningen Station and has proved to be preferable over the old blotter method.

4.8 Host-vigour and phoma lingam infection of cabbage seed

The behaviour of seed-borne *Phoma lingam* in cabbage is similar in many respects to the *Phoma* infections of corn-salad and beet.

For this infection the 2,4-D method is ordinarily used in many stations. The fact that pycnidia are formed more easily in the dark in the 2,4-D blotter test than in the normal one (de Tempe, 1962), seems to indicate the importance of the factor host vigour.

Some experiments were carried out with this infection to investigate the importance of host vigour. Although their results did show a certain tendency toward higher percentages with reduced host vigour (2,4-D and freezing), the available samples were too small and contained too little infection to reach definite conclusions.

A difficulty in the 2,4-D and freezing methods for *P. lingam* in cabbage seed is the frequent presence of saprophytic pycnidia-forming fungi – such as *Phoma herbarum* or *Peyronellaea glomerata* that also profit from the reduced host vigour. It is possible, however, to recognize *P. lingam* by its larger, often papillate or beaked pycnidia and by the bright amethyst to pink colour of its spore exudate.

The 2,4-D and the freezing methods seem to be the best ones for this infection as the agar method proved to be very unsatisfactory. In the first place the frequent presence of *Mucor* spp. makes NaClO pretreatment necessary, which was found seriously to reduce the *Phoma* percentage obtained. Secondly, the distinction between *P. lingam* colonies and those of similar saprophytic fungi is very difficult. For obtaining sporulation on PDA, incubation at 20°C in NUV light for at least 10 days was necessary. The exudate appeared as a rule only from the pycnidia on the seeds themselves. Colony characteristics are known to be quite variable, various strains of the fungus occurring (Pound, 1947).

When infected seed samples were sown in silversand and kept at 25 °C – the optimum temperature for disease development – only very little disease was found. The diseased plants developed lesions on the stem basis, on which pycnidia were produced when they were transferred to moist blotters and illuminated with NUV light. Similarly to what was observed for corn-salad, the roots of seemingly healthy cabbage seedlings proved to harbour the pathogen.

4.9 Host vigour and infections carried by wheat seed

For routine examination of commercial samples of cereal seeds, three incubation tests were used at the Wageningen Station, viz. one for *Helminthosporium* spp., one for *Fusarium* spp., and one for *Septoria nodorum*. In wheat seed *Helminthosporium* spp. are only rarely observed at Wageningen. The two other infections are prevalent and often reaching high percentages. In the blotter test for *Fusarium* spp. and in that for *S. nodorum* the recognition of seedling symptoms and not that of the fungus itself is most important. The recognition of these symptoms is not always easy and judgement is hard to standardize. Hence any modification of an existing method remedying this situation would be welcome. In the method for *Fusarium* spp., besides seedling symptoms, we also recognize a group of seeds with signs of infection. This is the usually small group of dead seeds with *Fusarium* mycelium.

The results with the *Phoma* infections described in the preceding sections led to experiments on host vigour in wheat.

4.9.1 *2,4-D experiments*

The effects of 2,4-D and of freezing were tried out first. If wheat seeds were incubated on blotters to which 0.2% 2,4-D had been added, the seedlings were stunted and did not become larger than about 1 cm. As inspection for symptoms of both diseases was now impossible, it was necessary to look for fungal characteristics. Also here a reduced host vigour might stimulate fungus development.

Many seeds or inhibited seedlings showed characteristic *Fusarium* or *S. nodorum* growth. The former can be recognized by colour and spore masses and the latter by greyish white curly mycelium, comparable to that obtained on agar. The data of table 52 show the results of such a 2,4-D experiment as compared with those obtained by normal blotter and agar tests. This experiment again indicated the importance of the factor host-vigour, by the proportionately larger

TABLE 52. Effect of the factor host vigour as shown by the average percentages of signs and symptoms of *Fusarium* spp. and *Septoria nodorum* found in 10 wheat seed samples by four different blotter tests and after NaClO pretreatment on PDA

testing conditions		*Fusarium*	*S. nodorum*
routine blotter method for *Fusarium*			
dead seeds + mycelium	(signs)	2.6	–
root browning	(symptoms)	38.9	–
routine blotter method for *Septoria*	(symptoms)	–	14.4
blotter test at 20°C in NUV light			
for 14 days	(signs)	2.5	0
idem, but with 2,4-D	(signs)	15.2	7.1
agar test (7 days at 20°C)	(signs)	26.6	14.7

percentage of seeds showing growth of the fungus itself, as compared with that in normal blotter tests. The total percentages obtained with the 2,4-D test were, however, on the low side.

The use of 2,4-D and maleic hydrazide in blotter tests with wheat seed was also tried by PONCHET (1960). This author found it to be satisfactory only for *S. nodorum*, for which he obtained about the same percentages as on malt agar.

4.9.2 *Experiments with the freezing method*

The effect of freezing was first tried out with seedlings, in analogy with the *Phoma* experiments of sections 4.5 to 4.8. The seedlings were obtained by keeping the tests first at 10°C for 3 days in order to break dormancy of the seed, and then for 2 days at 20°C in order to obtain rootlets of about 1 cm. The seedlings were then frozen overnight and subsequently incubated at 20°C in NUV light for another 5 to 7 days. *Fusarium* and *Septoria* grew well on the killed seeds and seedlings. Their development was more abundant than with 2,4-D and higher infection percentages were obtained. The rate of sporulation of the *Fusarium* spp. was much higher and hence their recognizability better. For *S. nodorum* this applied even more strongly. This latter fungus frequently produced large numbers of pycnidia from many of which spores were extruded in salmon-pink tendrils (Plate III, Fig. 2).

4.9.2.1 *Fusarium* spp. It appears from table 53 that the results of freezing were very good as far as total *Fusarium* and *Septaria* percentages were concerned. A comparison of these first results with those of the agar tests shows, however

TABLE 53. Comparison of average percentages for *Fusarium* spp. obtained by four different methods in 10 wheat seed samples

testing conditions	*F. nivale*	total *Fusarium*
routine blotter method for *Fusarium*	–	56.9
freezing method (3 days 10 °C + 2 days 20 °C followed by freezing, and then by 7 days 20 °C + NUV)	11.2	49.7
agar method without NaClO pretreatment (7 days at 20 °C on PDA)	17.5	48.4
agar method with NaClO pretreatment (7 days at 20 °C on PDA)	20.7	38.3

that *F. nivale* was insufficiently expressed by the given blotter test conditons. As this pathogen is very important, experiments were carried out to improve its chances. Two factors appeared to be important, viz a longer period of low temperature preceding the freezing and elimination of bacterial antagonism. After a prefreezing incubation of 7 days at 10 °C on blotters wetted with a 200 p.p.m. terramycin solution, and a postfreezing incubation on the same blotters of 5 to 7 days at 20 °C with NUV light, the best results were obtained (tables 54 and 55).

The *Fusarium* spp. were at first identified by making a spore preparation from each infected seed. This is quickly done by taking or scraping a little of

TABLE 54. Effect of the pre-freezing incubation regime on the average percentages of *Fusarium* spp. and *Septoria nodorum* found by the freezing method in 3 wheat seed samples. Agar test results are given for comparison

testing conditions	*F. nivale*	*F. graminearum* *F. culmorum*	*F. avenaceum*	*S. nodorum*
3 days 10 °C + 2 days 20 °C followed by deepfreezing, then 7 days 20 °C + NUV	11	4	10	34
7 days 10 °C followed by deepfreezing, then 7 days 20 °C + NUV	19	2	6	36
PDA after NaClO pretreatment 7 days at 20 °C	17	5	6	24

TABLE 55. Effect of four temperature regimes of the freezing method on the percentages of *Fusarium* spp. and *Septoria nodorum* found in 2 wheat seed samples. Agar test results are given for comparison

sample	method*	*F. nivale*	*F. graminearum* + *F. culmorum*	*F. avenaceum*	total *Fusarium*	*S. nodorum*
1	A	7.1	5.0	4.6	16.7	51.3
	B	10.8	7.1	10.6	28.5	65.7
	C	13.0	2.2	1.8	17.0	48.1
	D	13.7	4.3	3.5	21.5	67.4
	E	15.5	4.8	7.5	27.8	62.3
	F	12.8	7.8	11.5	39.1	56.8
2	A	11.5	5.2	7.7	24.4	1.1
	B	12.8	13.7	12.2	38.7	4.1
	C	17.9	2.8	3.8	23.5	2.0
	D	32.7	6.5	6.6	45.8	4.3
	E	34.3	7.3	7.8	49.4	2.5
	F	26.0	12.5	15.5	54.0	4.0

* A = 3 days 10 °C + 2 days 20 °C, followed by freezing, and then by 7 days 10 °C + NUV
B = as A, but last 7 days at 20 °C + NUV
C = 7 days 10 °C, followed by freezing, then 7 days 10 °C + NUV
E = on PDA for 7 days at 20 °C after NaClO pretreatment
F = as E, but without NaClO pretreatment

the spore mass with a needle and suspending it in water on a microscope slide or glass plate. The drops were not covered by cover slips but examined directly under the microscope at 100 × magnification.

After some time, when experience was gained in correlating appearance of the *Fusarium* on the seed with the identifications made, it was possible to immediately distinguish between the main *Fusarium* spp. under a stereoscopic microscope, with only occasional checks.

F. nivale, can be recognized by its salmon-coloured mycelium, which colour is intensified if it is scratched together with a needle. The effuse sporulation (pionnotes) along the hyphae often give rise to light orange-coloured small watery spore masses on the seed.

F. graminearum and *F. culmorum* can be identified by the typical yellowish and reddish mycelium with clearly distinct orange to red sporodochia.

F. avenaceum can be recognized by its large orange clod-like spore masses, readily apparent to the naked eye.

F. poae, finally, can be recognized by the fine white or pink cottony mycelium and its particular odour. At 25–50 × magnification the spores can be recognized as relatively small pale-coloured masses along the rather fine hyphae.

4.9.2.2 *Septoria nodorum*. With this infection the first freezing tests were carried out as already described in 4.9.2. *S. nodorum* can be recognized macroscopically by the typical mycelium on the seed and often also around it on the blotters (provided terramycin is used). The colony appearance is very much the same as that obtained on agar. Pycnidia can frequently be found on the seed surface, especially near the embryo end or on the coleoptyle. Here sporulation is most frequently observed, pink spore masses being extruded like toothpaste out of a tube, and often apparent to the naked eye. Sometimes it is necessary to scrape away some mycelium from the embryo end of the seed in order to detect the pycnidia more easily. The latter were also found on the rootlets, and in *Septoria* colonies on the filterpaper around the seed. Sometimes pycnidia were formed without much mycelium, as can also occur on agar. Then it was necessary to make sure that the pycnidia were of *Septoria*. When large spore tendrils are produced no problems exist, but if this is not the case confusion with the pycnidia of saprophytic *Phoma* spp. (notably *Phoma herbarum*) is possible. When neither typical *S. nodorum* mycelium is present, nor typical spore tendrils are produced, one should be very cautious. The pycnidia of *Phoma* and *Peyronelleae* are much smaller and more superficial, the spore exudate is usually much paler pinkish in colour and extruded in the form of a very thin short tendril or merely as a drop of exudate on top of the pycnidia. Of some saprophytic *Phoma* spp. the pycnidia easily fuse, forming a lobular outline and more than one stoma. The exudate consists of very small unicellular spores. These *Phoma* spp. become especially abundant if there are few other fungi present.

For 41 wheat seed samples of nearly one year old several methods were compared giving the average results of table 56. Those of the agar and freezing method were rather close, although the freezing results were somewhat lower. The results of the freezing method and the agar method with NaClO pretreatment gave a correlation coefficient of 0.86.

When these experiments were carried out *Mucor* became an increasing nuisance due to its gradual accumulation in the incubators. This air-borne 'laboratory weed' thrived on the killed seeds, rapidly spreading over the whole tray. It seriously hampered observation of the pathogens. In an attempt to

TABLE 56. Comparison of percentages of *Septoria nodorum* as found by four different methods in 41 wheat seed samples

testing conditions	*S. nodorum*
freezing method (3 days 10 °C + 2 days 20 °C, followed by deep-freezing, then 7 days 20 °C + NUV)	22.9
PDA, 7 days at 20 °C after NaClO pretreatment	28.3
idem, but without NaClO pretreatment	25.8

escape this problem blotter tests were made in NUV-transparent plastic petri-dishes. It turned out that in the dishes *Septoria* formed far less pycnidia and more mycelium. This mycelium is rather characteristic but as such less reliable than the pycnidia. The reduced fructification in covered plastic dishes suggested an insufficient illumination. In the incubators the NUV light is provided by vertical tubes (section 2.3.1). The light of the active wave lengths will be reduced passing through the quartz panes, usually covered with condensation and dirt. Especially the trays placed near the middle of the incubator will receive very little of the emitted radiation. The coverage of the seeds by the lids of the plastic petri-dishes or by polythene bags around trays apparently reduced the amount of NUV light received below the minimum required for a good fructification.

If the plastic petri-dishes with frozen seeds were placed on laboratory benches with NUV tubes horizontally above them at a height of 30 cm, excellent fructification and sporulation was again obtained. Illumination was given in cycles of 12 hours light and 12 hours darkness. When the lamps were on, the temperature near the seeds increased with 2 to 3 °C above the room temperature of 20 °C, which was measured by means of thermocouples. Later on the experiments were carried out in trays in a polythene bag supported by a wire frame. These so called 'tents' were also placed on laboratory benches underneath horizontal NUV tubes. The preceding may be illustrated by the data of a heavily infected wheat seed sample, given in table 57.

For *S. nodorum* also the elimination of bacterial growth improved the reliability of the method, the colonies growing more profusely on the seeds and even on the blotter around the seeds. Without terramycin many seeds had but little fungal growth, evidently due to bacterial antagonism.

With the non-optimal methods the observation was seriously hampered and the results were strongly fluctuating. Optimal conditions facilitate observation, which results in more reliable and higher figures.

TABLE 57. Effect of light intensity (NUV) on the percentage of clearly recognizable *Septoria nodorum* found by means of the freezing method in a wheat seed sample (The same sample gave 62% on PDA after NaClO pretreatment)

incubation in:	*S. nodorum*
open trays in incubator (20 °C)	67
closed plastic dishes in incubator (20 °C)	24
trays in polythene bags in incubator (20 °C)	60
closed plastic dishes under horizontal NUV tubes in laboratory room (about 20 °C)	79
trays in polythene bags under horizontal NUV tubes in laboratory room (about 20 °C)	79

The work on *Fusarium* had led to a temperature regime which was preferable for *F. nivale*. This regime consisted of 7 days incubation at 10 °C in darkness, then deep freezing overnight, followed by 7 days at 20°C under NUV light with 200 p.p.m. terramycin solution in the blotters. This regime proved also to be excellent for *S. nodorum* (cf. table 54 and 55).

4.9.2.3 *Helminthosporium* spp. *Helminthosporium* spp. occur only sparingly in Dutch wheat seed samples. It was found, however, that when present they always grew excellently on the frozen seeds, and were easily recognized. No accurate comparison with other methods was possible due to the low percentages found, but the impression was obtained that with this method *Helminthosporium* spp. were found more easily than with other methods.

4.10 HOST VIGOUR AND INFECTIONS OF OAT AND BARLEY SEEDS

In other cereal seeds *Fusarium* and *Helminthosporium* species also occur and may be important. With the freezing method *Septoria nodorum* was occasionally found in barley and even in oat seeds. On oats and also on barley and rye the growth of *Fusarium* spp. was less abundant than on wheat. Apparently the appearance of the fungi varies somewhat with the kind of seed tested. In general the sparse development of mycelium is an advantage.

It was interesting that perithecia of *Gibberella zeae* were frequently found, especially on oats in higher percentages than the imperfect state *F. graminearum*. Sometimes the seeds were covered by perithecia. These are black at first sight, but dark blue under high magnification, and sometimes oozing a dull-yellow

ascospore exudate. Later on they were also noticed on wheat seed, but to a lesser extent.

The shift between *Fusarium* spp. with changed temperature, as found for wheat seed, was also observed for oats and barley (tables 58 and 59). The tables also demonstrate that the old *Fusarium* blotter method for seeds of barley and oats gives far too low infection percentages. This may be due to the fact that it is very hard to judge the brown discoloration of the rootlets because of their own colour, which is often somewhat yellow to light brown.

In the case of the oat seed of table 58 the differences seem to be caused especially by *F. graminearum*. This fungus is known to be pathogenic for oats (McKAY, 1952). DE TEMPE (pers. comm.) checked this fungus for pathogenicity

TABLE 58. Comparison of average infection percentages found by various methods in 5 oat seed samples

method*	*Helminthosporium avenae*	*F. nivale*	*F. graminearum* *F. culmorum*	*Gibberella zeae*	*F. avenaceum*	*Fusarium* spp.	total *Fusarium*
A	–	–	–	–	–	–	13.8
B	27.0	–	–	–	–	–	–
C	33.2	3.6	27.6	–	3.0	1.4	35.6
D	46.8	2.0	30.4	20.6	6.2	3.4	62.0
E	42.4	3.4	18.8	23.4	4.4	1.6	52.6

* A = routine blotter method for *Fusarium* spp.
B = routine blotter method for *Helminthosporium* spp.
C = agar method after NaClO pretreatment (7 days at 20°C on PDA)
D = freezing method: 3 days 10°C + 2 days 20°C, followed by freezing then 7 days 20°C + NUV
E = freezing method: 7 days 10°C, followed by freezing, then 7 days 20°C + NUV

TABLE 59. Comparison of average infection percentages found by various methods in 5 barley seed samples

method cf. table 58	*H. sativum*	*Helminthosporium* spp.	*F. nivale*	*F. graminearum* + *F. culmorum*	*Gibberella zeae*	*F. avenaceum*	*Fusarium* spp.	total *Fusarium*
A	–	–	–	–	–	–	–	8.4
B	14.8	23.0	–	–	–	–	–	–
C	12.4	16.4	5.4	9.2	–	2.8	0.4	17.8
D	15.8	25.4	1.6	11.2	5.6	4.8	0.6	23.8
E	12.4	23.6	3.4	8.4	6.4	2.8	0.6	21.4

on oat seedlings by artificial infection of a relatively pathogen-free oat seed sample and found that in a soil test at 12 °C the control gave 87 % healthy plants and the inoculated one only 19 %. It was also interesting that when tested on blotters, the inoculated seeds gave only 18 % root symptoms. The freezing and the agar method (PDA without pretreatment) gave 90 % and 100 % respectively. Hence the blotter method is not very informative for *Fusarium* infection of oat seeds. The same is probably true for barley.

For *Helminthosporium* spp. the freezing method can be applied with different incubation regimes. The simplest one is keeping the seeds on moist blotters for 24 hours and then freezing them, for germination is unnecessary here. Five to seven days of post-freezing incubation at 20 °C with NUV light is sufficient for a rich sporulation of *H. avenae* and *H. sativum* on oats, and of *H. gramineum*, *H. teres*, *H. siccans* and *H. sativum* on barley. In many samples higher *Helminthosporium* percentages are found after freezing, which may be partly due to the increased ease of observation which renders the results more reliable.

In the Wageningen Station the blotter test, ordinarily used for seed-borne *Helminthosporium* spp. (10 days at 20 °C with NUV light) depends on the formation of conidiophores with conidia on the seed. Although the seedlings are cut off at inspection, yet the seeds are lying irregularly and at different levels, due to their being lifted by the roots. This makes inspection difficult and time-consuming and the results inaccurate. Thus the prevention of germination is an advantage. It is possible to use 2,4-D for this purpose, but this has drawbacks, as already mentioned (sections 4.2; 4.4).

4.11 Host vigour and infections of other kinds of seed

For other kinds of seed and their infections some experiments were carried out with the freezing method. This was only a survey to check the scope of the method. All the experiments were made by incubating seeds on blotters with 200 p.p.m. terramycin for 24 hours, followed by freezing and incubation at 20 °C in NUV light for 6 days or longer.

In pea seeds the percentages found for both *Ascochyta* spp. and *Stemphylium botryosum* were much higher than those of the old blotter test (5 days at 20 °C in darkness). A drawback for the routine use of the freezing method for this kind of seed is the abundant development of saprophytes. This can be reduced, but not entirely eliminated, by pretreatment with 1 % NaClO for 10 to 15 minutes and enclosing the trays in plastic bags. In the freezing method distinction between *A. pisi* and *A. pinodes* is difficult.

TABLE 60. Comparison of average infection percentages found by three methods in 10 pea seed samples

method	*Ascochyta pisi*	*Ascochyta pinodes*	*Stemphylium botryosum*
routine blotter method	9.9	4.9	1.4
freezing method (cf. text)	19.7	7.3	2.5
agar method (7 days at 20 °C on PDA)	19.2	8.8	3.9

As can be seen from table 60 the results of the freezing method were more or less of the same level as those of the agar method. In the latter the hypochlorite pretreatment takes much better care of the saprophytes (notably *Mucor* spp.) so that this method is cleaner and simpler and there fore preferable. If incubated on agar (PDA for at least 10 days, the colonies of *A. pinodes* can be distinguished from those of *A. pisi* by their dark colour (Plate III, Fig. 1).

In spinach seed somewhat higher percentages of *Colletotrichum spinaciae* and *Phoma betae* were found by the freezing method than in the normal blotter test.

For *Stemphylium radicinum* and *Alternaria dauci* in carrot seed the results of the freezing method are not higher than tose of the old blotter test with terramycin added, but are obtained with less effort. This makes them more reliable by reducing the personal factor in inspection. Here the freezing method offers an alternative to the use of 2,4-D, mentioned in section 4.2.

In poppy seeds higher figures were obtained for *Dendryphion penicillatum* than with the old blotter method. This was to be expected in view of the but only weakly pathogenic nature of this fungus (cf. MAAS GEESTERANUS, 1960).

Rice seed can carry a large number of pathogens. On the basis of the few samples available no definite idea could be formed, except for the fact that the freezing method rendered inspection quite easy. It probably increases the percentages of *Fusarium* spp. (*F. moniliforme* and *F. graminearum*), *Trichoconis padwickii*, *Phoma* spp. and *Pyricularia oryzae*, but perhaps not those of *Helminthosoporium oryzae* and *Curvularia* spp.

A number of samples of leek and onion seed received from the Plant Protection Service were examined by various methods for the presence of *Alternaria porri*. This important pathogen may be present in low percentages in some Dutch seed samples. These are not detected by the normal blotter test using NUV light. The agar test is also unsatisfactory as on agar the fungus is difficult to

recognize. With the freezing method the pathogen could occasionally be found and easily recognized by its beaked spores.

According to DE TEMPE (pers. comm.) the freezing method proved also useful for small-seeded legumes with *Phoma/Ascochyta*, *Botrytis cinerea*, *Fusarium* spp., and other fungi for seeds of endive with *Alternaria cichorii*, of Chinese aster with *Botrytis cinerea*, and of carnation with miscellaneous fungi.

4.12 THE PERSPEX-FREEZING METHOD

When working with the freezing method as a modification of the blotter method it was realized that certain inherent difficulties restricted its further development. It was especially the use of filterpaper that was considered to be an obstacle. The paper absorbs the nutrients leaching from the seeds and enables fungi to grow rapidly on and over it, thereby giving rise to secundary infections and increased interference by saprophytes. Another objection was the uneconomical way in which chemicals had to be applied. Much of them was lost to the paper and did not reach the surface where they should exert their effect. An example is the use of antibacterial antibiotics which are quite important for many freezing tests.

As germination is not essential, it was thought useful to supply the seeds with just sufficient moisture to enable initiation of the germination process and to allow freezing and fungal growth. After several trials the following procedure was developed.

Perspex plates of the normal blotter size and 6 mm thickness were used each with 75 holes of about 15 mm diameter and a few mm in depth. In each hole a sufficient amount of liquid could be placed together with one seed. After 24 hours or longer of preliminary incubation the seeds were killed by freezing at -20°C, as described in section 4.9 and further incubated under the desired conditions, usually at 20°C under NUV light.

Cereal seeds, with which the majority of these experiments were undertaken, require about 0.05 ml of water to which any desired chemical could be added. Terramycin was always added for restricting bacterial antagonism. The perspex plates were usually placed in a metal tray carrying a framework of about 5 cm high, and then sealed in a polythene bag. Some moist paper wadding underneath the plate served to maintain a high air humidity in these 'tents'. Such tents could also be placed at room temperature (around 20°C) underneath horizontal General Electric F 40 BL tubes, providing NUV as well as some visible blue light (Plate II, Fig. 1, 2 and 3; Plate III, Fig. 1).

For inspection the plates could be examined under a stereoscopic microscope. At the end of the test the holes were always dry, the seeds having absorbed all the available liquid. The distance between the holes was, however, insufficient for preventing spread of the fungi from seed to seed. Fungal hyphae running over the perspex could be observed. The data of table 61 show that this new modification gave results for wheat similar to those obtained by the agar method.

TABLE 61. Comparison of results of the perspex-freezing method with those of the agar method in terms of average percentages found for some *Fusarium* spp. and *Septoria nodorum* in 10 wheat seed samples

method	*F. nivale*	*F. graminearum* + *F. culmorum*	*F. avenaceum*	*S. nodorum*
perspex-freezing method	17.3	7.4	11.3	28.1
PDA after NaClO pretreatment	19.9	5.0	7.2	24.4
PDA without NaClO pretreatment	17.6	9.6	12.7	25.1

A disadvantage of the perspex plates is that they are expensive, whereas their cleaning is laborious. Hence for practical purposes one will have to turn to mass-produced vacuum-preformed plastic material that can be thrown away after use. This would render the method quite cheap. Some preliminary experiments with certain packaging materials (thin plastic sheet with depressions) supported the idea.

4.13 DISCUSSION

In this chapter another factor influencing seed health testing results was discussed, viz. host vigour. With many pathogens development on the host is made more easy if host vigour and thereby resistance to infection is lowered. For two of the three *Phoma* infections of the first sections of this chapter, the importance of host vigour is quite evident. Depending on the conditions the results obtained in a seed health test may be quite variable depending on seed or seedling vigour. It may be expected that the maximum percentage will be found after the host vigour has been reduced to zero, as is done in the blotter test with deepfreezing, provided that no other factor such as bacterial or fungal

antagonism interferes. Bacterial antagonism can be eliminated with antibiotics.

The percentages thus obtained are close to those on agar. Hence the difference between agar and normal blotter figures is due to host resistance. On agar host resistance does not play a role. So, the results can be expected to equal more or less those of the blotter tests with frozen hosts. Frozen seeds, especially if they are not first allowed to germinate, are comparable to agar medium in that they are just a quantity of nutrient material. If the viability of seeds is decreased, they tend to exudate more nutritive substances, such as sugars and amino acids. This seems to be especially true for dead seeds (TAKAYANAGI & MURAKAMI, 1968; cf. section 8.1).

The use of 2,4-D in seed health tests has its drawbacks. In the first place it is very persistent, so that all equipment used for 2,4-D tests has to be kept strictly apart. In a seed testing station it should never reach the germination department as it can cause serious difficulties there. In blotter tests the 2,4-D concentration used must be higher than in agar tests. Although the effect on fungi is probably less, its use implies the danger of influencing development of the fungi tested for (section 4.4). The 2,4-D results for cereals were moreover lower and more variable than those of the freezing method.

For *Helminthosporium* infections the easy inspection contributes to the increased percentages found, but a certain increase due to decreased host vigour may not be ruled out as more nutrients will be laching out from the seeds. This is a good example of the importance of rendering inspection easy. Not only does this tend to decrease costs, as it cuts down the time required for inspection and training, but it also makes the tests more dependable by reducing the influence of the personal factor (analyst).

For *Fusarium* spp. and *Septoria nodorum* in wheat and other cereals, the freezing method is an interesting one. If precautions against air-borne contaminants, notably *Mucor* spp., are taken, these pathogens are easily determined in the freezing method although experience is necessary. With *Fusarium* it is even possible to distinguish roughly between the main species. Freezing of young wheat plants was also practised by BECKER (1963) in proving the seed transmission of *S. nodorum*.

The freezing method is also useful in finding trace infections of important seed-borne pathogens, such as *Didymella lycopersici* in tomato seeds and *Alternaria porri* in seeds of onion and leek.

Contrary to 2,4-D, which may have harmful effects on fungi, freezing was never found to affect the latter in any way. Excess lenght of the freezing period was of no consequence. A certain minimum time was required which varied

with the seed and other circumstances, such as the load of the freezer, the amount of ice formation in the freezer, etc. For safety's sake the experiments were always left in the freezer overnight, but a few hours may suffice. So the length of the freezing period can be adjusted to the time schedule of the analyst.

The deep-freezing method can be easily introduced into seed testing stations as a deep-freezer is not expensive. A vertical model with a number of independent lockers appears to be most convenient. It would be practical to adapt the size of the lockers to those of the standard racks for blotter trays.

At Wageningen the freezing method is now used in routine testing of commercial samples of cereals and grasses for *Helminthosporium* spp., carrots for *Stemphylium* and *Alternaria* spp., cabbage for *Phoma lingam* and many other infections of small seeds.

5 INTERFUNGAL ANTAGONISM

5.1 INTRODUCTION

In the preceding chapters interfungal antagonism was encountered already quite frequently. It is mainly caused by the presence of other fungi besides the pathogen on or in the tested seeds.

It can be said that the less important the factor host vigour, the more copious is fungus growth and the more important is this type of antagonism. So it plays a greater role in the agar and freezing methods than in the ordinary blotter method. The antagonism encountered may be of various types (cf. PORTER, 1923), but the most frequent one is that caused by competition for food. The pathogen can be outgrown by its saprophytic competitors.

For the purpose of seed health testing it is important that the organisms tested for should be as easily noted as possible. When a pathogen is overgrown or restricted by other fungi, but still recognizable, detection is more difficult. This is undesirable for routine tests as it makes them more time-consuming and costly, and less accurate.

In this chapter some of the work done on this factor will be discussed.

5.2 THE EFFECT OF TEMPERATURE

It was already made clear in chapter 3 that antagonism between fungi and bacteria is influenced by incubation conditions. The same applies to interfungal

antagonism. Some conditions are more favourable for certain fungi than for others and hence the fungi will be differentially affected. Very often, however, optimal conditions are the same or very similar for many fungi.
Such optimal conditions will favour those species that are the strongest growers or those that make the best start.

This can be illustrated with the observations made on the effect of temperature on the blotter test for wheat seed. Lowering of the incubation temperature caused an increase of severe symptoms. When 74 wheat seed samples were tested on blotters for *Fusarium* spp. with two incubation regimes (3 days at 10 °C, followed by either 3 days at 20 °C as normally used, or by 3 days at 15 °C), it was found that with the second regime the number of severe symptoms doubled at the expense of the light ones (table 62). The total infection percentage did not change.

TABLE 62. Effect of temperature during the last 3 days of incubation on the average percentages of symptoms found in the routine blotter test for *Fusarium* spp. in 74 samples of wheat seed.

1	2	3	4	5	6	7	8
temperature regime*	dead seeds with *Fusarium*	severe root rot symptoms	light root rot symptoms	epiblast browning	abnormal seedlings	dead seeds	sum of 2+3+4 = total *Fusarium*
I	1.6	2.5	16.7	14.7	2.9	2.3	20.8
II	1.6	5.0	14.7	10.2	4.3	2.4	21.3

* I = 3 days 10 °C, followed by 3 days 20 °C
II = 3 days 10 °C, followed by 3 days 15 °C

It was also tried to make blotter tests at still lower temperatures, such as 7 days at 12 °C and 14 days at 10 °C, but this resulted in too much interference by *Septoria nodorum*. The abnormality of seedlings was, however, very much increased in this way, light infections no longer being found. It could be proved by means of agar platings (table 63) and correlation figures (table 64) that this increase was due to *F. nivale*. The shift toward *F. nivale* was also repeatedly noted in freezing tests. Here the prefreezing incubation temperature influenced the proportion between *F. nivale* and other *Fusarium* spp. found (cf. sections 4.9 and 4.10). In the field *F. nivale* (the snow-mould) is also known to be favoured by low temperatures. In soil tests we found it necessary to keep the temperature below 15 °C in order to obtain any disease symptoms.

TABLE 63. Analysis of symptoms found in routine blotter tests for *Fusarium* carried out at two temperature regimes for two wheat seed samples, by plating of diseased rootlets on PDA

sample*	blotter test			number of times *Fusarium* spp. were isolated on PDA		
	temperature regime**	symptoms	number of times symptoms occurred per 400 seeds	*F. nivale*	*F. graminearum* + *F. culmorum*	*F. avenaceum*
A	I	dead + *Fusarium*	7	0	7	0
		severe	22	11	7	0
		light	164	140	6	1
	II	dead + *Fusarium*	5	0	5	0
		severe	47	37	9	0
		light	141	127	7	0
B	I	dead + *Fusarium*	8	0	6	2
		severe	34	2	29	0
		light	109	37	23	11
	II	dead + *Fusarium*	8	0	8	0
		severe	53	9	3	0
		light	87	36	25	9

* The two samples gave on PDA:

sample	NaClO pretreatment	*F. nivale*	*F. graminearum* *F. culmorum*	*F. avenaceum*
A	–	44	7	1
	+	43	3	1
B	–	9	27	11
	+	11	15	5

** cf. table 62

It was found that seedlings that were attacked by the fungus in the blotter test (root browning), did for the larger part produce healthy plants when transplanted into ordinary soil at 15 °C. Even seedlings with severe symptoms usually recovered. The same was not the case, however, after transplantation into silversand at 7 °C. This experiment was carried out on small round white blotter discs (about 8 mm in diameter) laying on moist silversand. The paper was necessary for the observation of the root symptoms and the silversand for transplantation without damage (cf. table 65).

In the agar method *F. nivale* tends to be easily overgrown by other fungi,

TABLE 64. Correlation coefficients between severe and light symptoms in the *Fusarium* blotter test at two temperature regimes for 74 wheat seed samples, and the percentages of *F. nivale* and *F. graminearum* as found on PDA, with and without NaClO pretreatment
(Coefficients of 0.25 and higher are significant on P = 95% level; those of 0.38 and higher on P = 99% level)

temperature regime*	blotter test symptoms	agar test results for			
		F. nivale		*F. graminearum*	
		with NaClO	without NaClO	with NaClO	without NaClO
I	severe	0.26	0.25	0.59	0.59
	light	0.67	0.60	0.13	0.23
II	severe	0.54	0.50	0.30	0.32
	light	0.64	0.66	0.15	0.26

* cf. table 62

TABLE 65. Effect of transplating of wheat seedlings showing root browning symptoms in the routine blotter test for *Fusarium* to silversand and greenhouse-soil at two temperatures. The blotter test had been carried out with each seed on a small paperdisc on top of silversand (cf. text).
The wheat seed sample used gave on PDA after NaClO pretreatment: 34% *F. nivale*, 1% *F. graminearum*, 2% *S. nodorum*

experiment in:	medium	temperature	blottertest symptoms used	percentage giving rise to:		
				plants without coleoptyle lesions	plants with coleoptyle lesions	abnormal or dying plants
climate cell	silversand	7°C	severe	0	86	14
			light	60	38	2
	soil	7°C	severe	69	28	3
			light	91	3	6
	silversand	15°C	severe	89	4	7
			light	97	3	0
	soil	15°C	severe	94	3	3
			light	100	0	0
greenhouse	soil	15°C	severe	95	2	3
			light	100	0	0

especially in the beginning. This makes it to be overlooked easily, as it produces rather flat thin colonies which are hard to identify with certainty. The fungus has a high growth rate and it therefore often grows out from under the edge of other colonies filling the spaces between them. This hampers inspection and introduces errors of judgment. The same applies to the counting of colonies of fungi that tend to merge without distinct borderline, which makes it hard to tell the number of seeds from which colonies arose. This is, e.g., often the case with *F. nivale*. Later on the appearance of the colonies is usually more distinctive, those of *F. nivale* having the tendency of turning salmon in colour and often producing small orange spore masses where rootlets happen to penetrate the agar.

The question that presented itself was whether interference by other fungi could be reduced by changing the incubation temperature of the agar test. Wheat seeds harbouring fungi in various proportions were plated out on PDA in the normal manner and incubated at 3 different temperature regimes, viz. 7 days at 20°C, 10 days at 15°C, and 14 days at 10°C. Colonies arising from these seeds were given a number on the reverse side of the plate. They were measured daily, starting from the third day. After identification of the colonies at the end of the experiments, their average diameter was calculated for all fungal species present and plotted against time. Those for the main ones are given in fig. 5.

It follows that *F. nivale* favours 20°C but that at the lower temperature its growth is proportionately less reduced than that of the other *Fusarium* spp. *F. nivale* is at an advantage at lower temperatures as far as competitive ability is concerned. On agar the use of lower temperatures perhaps makes the recognition of *F. nivale* easier but does not result in higher percentages obtained (table 66) and it requires a longer period of incubation.

At lower temperatures in the blotter method and in soil, *F. nivale* is likely to

TABLE 66. Effect of incubation temperature on the average percentages of some seed-borne fungi growing from 4 wheat seed samples on PDA after pretreatment with 1% NaClO for 10 minutes

length of incubation in days	incubation temperature	*F. nivale*	*F. graminearum*	*F. avenaceum*	total *Fusarium*	*Septoria nodorum*	*Epicoccum*	*Alternaria*
14	10°C	22	10	4	36	5	24	21
10	15°C	20	8	5	33	7	25	24
7	20°C	23	11	5	39	8	25	25

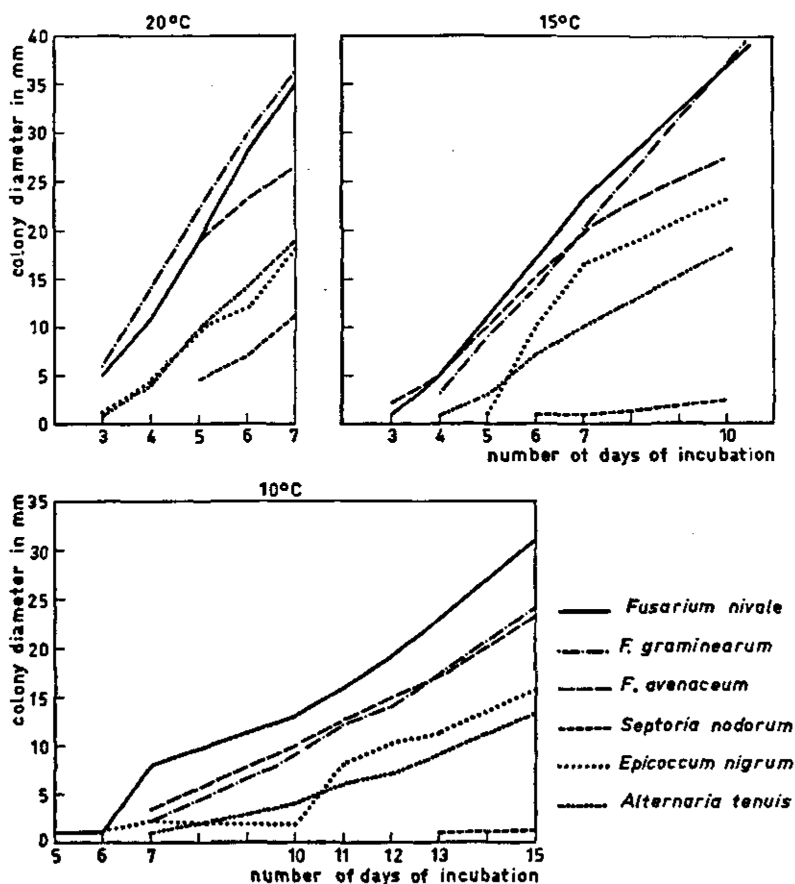


FIG. 5. Effect of 3 different temperatures on the growth-rate of some fungi developing from wheat seeds on PDA (see text)

get into a better position due to the fact that the reduced growth of its antagonists enables it to profit from its parasitic capacities.

It may be of interest in this connection that ENDO (1963), reported an optimum temperature for growth of *F. nivale* on PDA of 20 °C. At this temperature *F. nivale* proved to be able to attack bentgrass seedlings growing in sterile silversand, which it does not do in the field.

Septoria nodorum is a relatively slow grower on PDA even at 20°C, but at 15 and especially at 10 °C its growth becomes very slow so that the latter two temperatures cannot be recommended for agar tests for this pathogen either.

5.3 THE USE AND EFFECT OF SUPERFICIAL DISINFECTION WITH HYPOCHLORITE

The common occurrence of interfungal antagonism in agar tests has led to the general use of surface pretreatment. The reasoning behind it is that the pathogen is supposed to be located deeper inside the host tissue than many of the saprophytes.

Several disinfectants can be used such as H_2O_2, $HgCl_2$, formalin and ethyl alcohol, but in practice the use of hypochlorite solutions has been found most satisfactory. It has several advantages such as being harmless and not unpleasant to work with and leaving no residues on the seed. The difficulty of standardization can be overcome by the use of concentrated stock solutions containing stabilizers (e.g. sodium metasilicate). They should be stored in dark and well-closed containers. Working solution should always be fershly made. The disinfection itself is carried out by immersing the seeds e.g. for 10 minutes in a ½ or 1% NaClO solution after which the seeds are drained off and transferred immediately unto the agar surface by means of flamed forceps.

TABLE 67. Effect of NaClO pretreatment on the average results of agar tests for a number of fungi found in 74 wheat seed samples of the 1964 harvest and 136 samples of the 1965 harvest

main fungi recorded	1964 harvest		1965 harvest	
	without NaClO	with NaClO	without NaClO	with NaClO
Fusarium nivale	16.0	16.2	12.8	16.2
F. graminearum	7.9	4.5	3.8	1.8
F. culmorum	0.2	0.1	1.0	0.1
F. avenaceum	1.2	1.1	6.9	2.5
F. poae	0.6	0.7	0.3	0.4
Fusarium spp.	0.1	0.2	0.2	0.6
total *Fusarium*	25.9	22.6	24.8	21.0
Septoria nodorum	9.2	7.3	6.8	8.8
Helminthosporium spp.	1.5	1.5	1.2	1.0
Epicoccum nigrum	44.7	25.2	65.1	25.9
Alternaria tenuis	47.5	43.0	46.8	38.0
Stemphylium botryosum	2.6	3.0	3.1	4.5
Botrytis cinerea	4.0	3.3	3.5	2.4
Penicillium spp.	23.3	0.4	22.5	0.7
Acremoniella atra	1.4	0.1	3.5	0.7

In section 3.19 we noted the reducing effect of NaClO pretreatment on the bacterial seed flora, but we have also seen that the use of antibiotics in agar is a much more efficient way of taking care of them but this does not reduce the saprophytic fungi. It is evident that surface disinfection does reduce the percentage of saprophytic fungi. The immediate effect it has on the pathogenic fungi to be determined is less clear. In order to get some insight into this matter many samples were tested with and without NaClO pretreatment.
Table 67 gives the average results for respectively 74 and 136 wheat seed samples tested in two consecutive years. It can be observed that whereas the percentage of *F. nivale* tended to remain constant or to increase after NaClO pretreatment, that of other *Fusarium* spp. was reduced by half. This reduction was observed throughout in the samples tested. As *F. graminearum* and *F. culmorum* are important pathogens this reduction was considered objectionable. *F. nivale* gave the same or higher percentages at an average, combined with easier observability, but this did not apply to each sample. There were a few samples in which it did decrease after surface disinfection, but normally the effect of the elimination of fungal competitors more than balanced the loss it suffered itself by the treatment and this indirect effect prevailed in the averages.

It was noted that the samples in which the percentage of *F. nivale* decreased were always those containing relatively few other fungi, notably *Epicoccum nigrum*. Table 68 illustrated this for a few representative samples. It can be noted that the percentage of *F. nivale* is decreased by the NaClO pretreatment in case the chemical has little effect on *Epicoccum*, but that the *F. nivale* percentage is increased if the percentage of *Epicoccum* is greatly reduced. The effect of the presence of some other fungi which by their rapid growth cover the plate and prevent development and observation of e.g. *F. nivale* is evident.

TABLE 68. Relation between the effects of NaClO pretreatment on the percentages of *Epicoccum nigrum* and *Fusarium nivale* found on PDA with 4 selected representative wheat seed samples

sample	*Fusarium nivale*		*Epicoccum nigrum*	
	without NaClO	with NaClO	without NaClO	with NaClO
1	55	39	14	6
2	14	19	59	13
3	12	35	94	28
4	6	37	89	32

The same is true for other competitive fungi, such as *F. graminearum*, *Botrytis cinerea* and *Mucor* spp.

The different behaviour of *F. nivale* and, e.g., *F. graminearum* can be explained by the fact that the first is very sensitive to competition and is also easily masked whereas the latter is a better competitor and not easily masked. In section 5.6 the effect of the NaClO pretreatment on the *Fusarium* spp. will be discussed again, namely in connection with its use in a method selective for *Fusarium* spp. In this specific method the NaClO pretreatment always reduced the percentage of *Fusarium* found.

The NaClO pretreatment decreased the amount of *Septoria nodorum* somewhat in the first and increased it in the second year (table 67). There was, however, a lot of difference between samples, some yielding an increased, others a decreased percentage. Here more or less the same applies as what has been said for *F. nivale*. In contrast to *F. nivale*, however, *S. nodorum* grows slowly. In agar tests it may find its place occupied by others and is easily masked, especially by *Epicoccum*. We will return to this subject for *S. nodorum* in section 5.7.

In an experiment in which petri-plates with PDA were inoculated with *S. nodorum* on one side and with another fungus on the other, it was found that the other fungus after reaching the *Septoria* colony would grow around it and prevent it from growing any further. An interesting observation made in this way was also that as soon as the other fungi reached *Septoria*, the latter changed colour from more or less yellow to olive-brown or -green.

Tables 69, 70 and 71 show that in the blotter and freezing methods NaClO pretreatment always reduced the percentages found. The same applies to sand and soil tests. To this matter we will return in section 6.2.

In case of testing for *Helminthosporium* spp. in cereals and grasses exactly

TABLE 69. Effect of NaClO pretreatment on the average results of *Fusarium* blotter test for 136 wheat seed samples of the 1965 harvest

category	without NaClO	with NaClO
dead seeds with *Fusarium*	2.8	1.6
severe rootrot	4.2	3.2
light root rot	21.6	13.7
total *Fusarium*	28.6	18.5
epiblast browning	4.0	2.6
abnormal seedlings	5.4	6.2
dead seeds	4.4	5.8

Table 70. Effect of NaClO pretreatment on the average results of the routine blotter test for *Septoria nodorum* for 20 wheat seed samples

without NaClO	with NaClO
16.1	7.2

Table 71. Effect of NaClO pretreatment on the average pathogen percentages found in 10 wheat seed samples with the perspex-freezing method

pathogen	without NaClO	with NaClO
Fusarium nivale	13.6	9.0
F. graminearum + *F. culmorum*	5.6	2.4
F. avenaceum	3.5	0.9
Septoria nodorum	32.0	21.6

the same phenomena can be noted. In the blotter and freezing methods the *Helminthosporium* percentage is always decreased by the pretreatment. In the agar method the average percentage is more or less the same or somewhat increased. If one studies the effect on individual samples, however, many show a decrease. These were always samples with a relatively high infection percentage and few other fungi present. The agar method is therefore less satisfactory for these infections.

The same is true for *Stemphylium radicinum* and *Alternaria dauci*, for which two representative examples are given in table 72 (cf. also Hewett, 1964).

The results of these investigations into the effect of the pretreatment on the surface flora clearly demonstrate that this way of combating interfungal antagonism is a very arbitrary one. Only deeper seated infections are not decreased

Table 72. Effect of NaClO pretreatment on the pathogen percentages found in blotter and agar test (PDA) with two carrot seed samples

sample	pathogen	blotter method		agar method (7 days 20 °C on PDA)	
		without NaClO	with NaClO	without NaClO	with NaClO
1	*Stemphylium radicinum*	52¼	8¾	41	7¾
	Alternaria dauci	¾	0	0	0
2	*Stemphylium radicinum*	27⅓	14	20⅓	11¼
	Alternaria dauci	59	22¼	37⅓	24¾

by it. An example of that is *Ascochyta pisi* in pea seed. NaClO pretreatment was not found to affect the *Ascochyta* percentage obtained in the blotter test. In the agar and feezing method it is an absolute necessity to use NaClO because otherwise *Mucor*, which is always present, dominates. Another example is the *Phoma betae* infection of beet seed, in which the pathogen has penetrated deeply into the cluster tissues (section 5.4). The effect of NaClO pretreatment on this infection is clear from the data of tables 48, 50 and 73. Here the effect in the agar test is an increasing one. In the blotter test NaClO usually reduces the *Phoma* percentage found. The reason for this difference is probably the same as that for *Fusarium* and *Septoria* blotter tests of wheat, namely a matter of inoculum potential (cf. ch. 6).

The effect of surface pretreatment is, consequently, somewhat complex and it differs with the infections studied and even with the seed sample investigated. It is clear that more specific methods of reducing the effect of interfungal antagonism would be preferable. It also shows the advantage of the blotter and freezing method for such infections as *Helminthosporium* spp. in cereals and grasses, and of *Stemphylium radicinum* and *Alternaria porri* in carrot seed. It is one more reason for the differences in percentages obtained by agar and blotter methods, interfungal antagonism being less important in the blotter method.

5.4 The effect of removal of external 'seed' structures

In section 4.5 and table 48 we saw that the percentages of *Phoma valerianellae* obtained on PDA with seeds liberated from their fruit structures were higher than the percentages in the non-dissected fruits. This can be explained by the fact that by taking away the fruit structures most fungal antagonists were removed as well, for the free seeds yielded only *P. valerianellae*. The removal was carried out by hand. Although it was later found that it could be done more quickly mechanically by the seed cleaning department, this implied the danger of external contamination by the produced dust. NaClO pretreatment of isolated seeds considerably reduced the percentage of *Phoma*.

The same approach was tried for *Phoma betae* is beet seed clusters, but here it proved very difficult to isolate intact seeds from them. Isolated seeds frequently yielded the pathogen in blotter and agar tests, proving that they were often infected. Due to the above difficulty no quantitative data could be obtained.

The effect of removing the outer corky layer of the clusters was also tried. This was carried out in a rubbing machine of the cleaning department. The results were hard to interprete because of the influence of outward contamina-

tion by dust produced by the rubbing and because germination was seriously impaired (with 30%). The first problem was coped with by washing the seeds. The second was more difficult but led to the idea of using the production of pycnidia on the cluster tissue itself as criterion. This was incidentally found to be stimulated by NUV light. This led to the development of a new blotter method described in section 4.6. With this method only two samples were tested. For the first sample NaClO reduced the percentage of *Phoma* from 53 to 41, and in the second from 64 to 49 (800 seeds being used per object). Agar tests showed a reduction of the flora obtained, notably of *Mucor* spp., *Cephalothecicum roseum*, *Alternaria* spp., *Acremoniella atra*, *Fusarium avenaceum*, etc., but also *Phoma* itself. The average *P. betae* percentages for 10 samples with 800 seeds per object are given for rubbed and non-rubbed beet seed clusters after different pretreatments as indicated (table 73). The results show that after rubbing bacterial antagonism has disappeared. It seems to be mainly present in the outer tissues. NaClO pretreatment does not clearly reduce the *Phoma* percentage found but the number of saprophytic fungi on PDA was always reduced to a low and non-troublesome level. Vacuum or ethyl alcohol dip promotes the penetration of NaClO into the outer corky layers which contain many air spaces. In the rubbed samples the effect on the remaining woody part is much less. The data suggest that a normal pretreatment of the clusters with 1% NaClO for 10 or 15 minutes followed by agar testing will give the best information (cf. section 4.7).

Many cereal and grass seeds possess glumes, which are dead tissues always harbouring many saprophytes. It was found that their removal nearly always

TABLE 73. Effect of various pretreatments on the average percentages of *Phoma betae* found on PDA for 10 beet seed cluster samples (rubbed and non-rubbed, cf. text)

pretreatment	whole clusters	rubbed clusters
none	69	53
15 minutes 1% NaClO	71	50
idem, but after alcohol dip for 30 seconds	49	46
15 minutes 1% NaClO under vacuum	59	47
24 hours water soak	53	55
24 hours soak in 100 p.p.m. terramycin	79	51
idem, but after vacuum infiltration	82	56
24 hours soak in 100 p.p.m. streptomycin after vacuum infiltration	78	47

results in reduced pathogen percentages with the possible exception of *Fusarium nivale* (table 74, 75, 76). The latter fungus seemed even to increase, probably due to diminished competition.

Table 77 shows the effects of dehulling of 4 oat seed samples on results on blotters, on agar and in silversand, and the importance of the glume infection for disease development. Discarding the hulls apparently is not warranted. In section 6.4 an example will be given of *Helminthosporium gramineanum* in barley seed where the hull infection appeared to be prevalent but unimportant. The effect of dehulling in soil tests is complicated as it involves the removal of possible antagonists present in the glumes as well as lowering of the inoculum potential (cf. ch. 6). For practical purposes dehulling would, moreover, be a time-consuming procedure. The use of NaClO pretreatment is to be preferred.

TABLE 74. Effect of glume removal on the percentages of *Helminthosporium* spp. found in the blotter test with some kinds of seed

seed	pathogen	number of samples	control	dehulled
grasses	*Helminthosporium* spp.	4	21.0	6.2
oats	*H. avenae*	4	67.3	44.3
barley	*H. siccans*	4	27.8	20.0
	H. sativum	2	13.5	6.0

TABLE 75. Effect of glume removal on percentages of various fungi found on PDA in an oat seed sample

fungi recorded	seeds with glumes		glumes removed	
	without NaClO	with NaClO	without NaClO	with NaClO
Fusarium nivale	3	2	3½	9
F. graminearum/culmorum	6½	2	2½	0
F. avenaceum	3	½	0	0
Fusarium spp.	½	1	0	0
Helminthosporium avenae	47	42	38	27½
Epicoccum nigrum	28½	13	17	6½
Alternaria tenuis	76½	68½	60	51½
Stemphylium botryosum	1	4	2½	6
Cladosporium herbarum	5	0	2	0
Botrytis cinerea	1½	2	2	½
Acremoniella atra	1½	0	0	0
Stachybotrys atra	½	0	0	0
Penicillium spp.	4	0	3	0
Mucor spp.	2	0	3	0

TABLE 76. Effect of glume removal of barley seeds* on percentages of various fungi found on PDA

fungi recorded	seeds with glumes		glumes removed	
	without NaClO	with NaClO	without NaClO	with NaClO
Fusarium nivale	10	10½	12	17½
F. avenaceum	5½	1½	1	0
Helminthosporium sativum	4½	3	2½	1
H. siccans	28½	23½	22	17½
Epicoccum nigrum	35	14	11	7
Alternaria tenuis	57	43½	36	21½
Stemphylium botryosum	4½	8	6½	5
Botrytis cinerea	0	2	0	0
Acremoniella atra	8½	0	½	0
Mucor spp.	6	0	2	0

* blotter tests with this sample gave respectively 37½ % *H. siccans* and 4% *H. sativum* for the control, and 18½ and ½ % for the dehulled seeds

TABLE 77. Effect of removal of glumes from oat seeds on the percentage of *Helminthosporium avenae* subsequently found in blotter and agar tests as well as the percentage disease found in silversand at 12 °C

sample	blotter test		agar test (PDA)				disease in silversand	
	with glumes	without glumes	with glumes		without glumes		with glumes	without glumes
			without NaClO	with NaClO	without NaClO	with NaClO		
1	62½	48	42	47	43½	36	16½	11
2	71	47	32	46	36	29	21	14
3	89½	66½	61½	70½	64	32½	27	11½
4	46	14½	23	21½	9½	10½	9	7½

5.5 THE DRY HEAT TREATMENT

MALONE (1962) discovered a very elegant way for combating interfungal antanism in agar tests for *Helminthosporium avenae* in oat seeds. He kept the seeds at 100 °C for one hour and found that this did not affect *H. avenae*, whereas it reduced some other fungi, notably *Alternaria tenuis*. The latter is a nuisance when searching for *H. avenae*, as it masks the characteristic growth of the pathogen when both occur together.

The present author applied the Malone method with similar results not only

for *H. avenae* but also for *H. sativum* in oat seed, as well as for *H. sativum*, *H. siccans*, *H. teres*, and *H. gramineum* in barley seed. Its application to *Helminthosporium* spp. in grass seeds was less successful (table 78).

Unfortunately, however, *H. avenae* is not the only fungus on the seed resistant to the dry heat treatment. Interesting is the increased percentage found for the red yeast-like fungus *Sporobolomyces roseus* from just a few percent of small pinpoint sized colonies to nearly 100 % of much larger colonies in some samples. They do not interfere with the determination of *H. avenae* and consequently

TABLE 78. Effect of dry heat treatment (one hour at 100 °C) and NaClO pretreatment on the results of agar, blotter and freezing tests for *Helminthosporium* spp. of various cereal and grass seed samples

host and pathogen	agar method (PDA)			blotter method			freezing method		
	control	NaClO	100 °C	control	NaClO	100 °C	control	NaClO	100 °C
1 oats									
H. avenae	50	58	89	45	37	86	78	67	97
2 barley									
H. gramineum	87	89	94	78	42	94	96	96	92
3 barley									
H. gramineum	17	7	19	13	20	16	34	13	36
4 barley									
H. gramineum	74	62	67	71	48	63	73	64	63
5 barley									
H. sativum	93	95	93	88	94	98	100	96	96
6 barley									
H. sativum	28	23	49	29	26	47	28	23	46
H. siccans	0	3	0	1	2	8	6	4	10
7 barley									
H. sativum	3	0	0	0	0	0	0	0	0
H. siccans	20	37	27	35	36	44	60	46	62
8 barley									
H. sativum	80	70	95	83	65	97	90	70	88
H. siccans	0	0	4	0	1	2	0	4	10
9 perennial ryegrass									
H. siccans	2	0	14	27	6	9	29	8	12
10 perennial ryegrass									
H. siccans	0	1	6	29	4	16	26	5	17
H. dictyoides	0	0	0	5	0	0	7	1	0

are of no practical concern. The latter is the case, however, for the likewise increased percentage found for *Mucor* spp. Apparently weak *Mucor* infections are present on many seeds as well but do not play a role due to competition by other fungi. This recalls the *Mucor* problem in the freezing method for pea seeds, even after NaClO pretreatment. As the presence of even one *Mucor* colony may spoil a plate, this presents a serious drawback for the dry heat method.

It was then decided to try the treatment for blotter tests as well. The results were excellent as *Mucor* was no problem here. Inspection of the seeds was rendered easy by the almost total absence of *Alternaria tenuis*. Nearly all fungal structures observed are *Helminthosporium* spp. Seed germination was much inhibited which also simplified inspection.

Table 78 gives an idea of the effect of the heat treatment and NaClO treatment in respectively the agar, the blotter and the freezing method. It again clearly shows the reducing effect of NaClO on the percentage of infection found. The heat treatment does not have this effect. Freezing method and blotter method with dry heat treatment are more or less equivalent. The dry heat treatment before the freezing method simplifies its inspection perhaps a little more but has no further clear advantages.

As in blotter tests after dry heat treatment the sporophores were sometimes

TABLE 79. Effect of moisture content of oat and barley seed on the results of the dry heat treatment

sample	moisture content	non-heated control			1 hour at 100 °C		
		H. avenae	*H. sativum*	*H. siccans*	*H. avenae*	*H. sativum*	*H. siccans*
oats 1	11.7	66	2½	–	92½	1½	–
	14.3	61½	2½	–	90½	2	–
	16.9	66	1½	–	30	0	–
	19.7	67½	1	–	38	0	–
oats 2	11.7	39	4	–	53½	7½	–
	14.6	44½	6½	–	58	3½	–
	17.3	37½	4½	–	23	½	–
	20.0	45	7½	–	2½	½	–
barley 1	13.6	–	½	27½	–	½	38½
	15.1	–	1	29½	–	½	35
	17.8	–	1	29	–	0	5
	20.7	–	0	25½	–	0	0
barley 2	15.3	–	64	½	–	61½	6½
	16.4	–	59	2½	–	69	4½
	18.6	–	66	1	–	5½	0
	21.4	–	60½	3	–	1½	0

less abundant and of poor shape, it was assumed that the heat had been too severe for them. Possibly moisture content variations of the seed might be of influence. To investigate the level above which *Helminthosporium* would start to be affected, an experiment was carried out with two oat and two barley seed samples at different levels of moisture content. The level and the results are given in table 79. The figures show that the limit lies around a moisture content of 15%. Therefore it is necessary to make sure that the samples treated in this way are dry enough; if not, they should be dried beforehand.

In grass seed shorter periods of dry heat treatment were found to give better survival of *H. siccans*, but *H. dictyoides* is already seriously affected after 15 minutes (table 80).

TABLE 80. Effect of dry heat treatment on the percentage of *Helminthosporium* spp. found in the blotter test for 2 samples of perennial ryegrass

sample	pathogen	length of dry heat treatment (100 °C) on minutes			
		0	15	30	60
1	*H. siccans*	26½	24	30½	17½
	H. dictyoides	34	24⅓	10	2⅓
2	*H. siccans*	17½	20	21½	14
	H. dictyoides	40	26	23	16

5.6 EXPERIMENTS WITH SELECTIVE COMPOUNDS

A first attempt toward selective methods in seed health testing was made by PONCHET (1960). He examined among others the effect of low concentrations of $CuSO_4$ and $HgCl_2$ in malt agar on the percentages found for fungi growing from wheat seed. Interesting differential effects were obtained but not sufficient for routine application. KULIK & COMBE (1967) tried to develop selective culture media based on nutritional requirements of seed-borne fungi but were unable to separate pathogens and saprophytes.

In soil microbiology selective media are frequently used for the isolation of specific organisms from soils. The number of such procedures has continuously increased over the last years, most of them making use of selective chemicals. A distinction can be made between compounds which selectively eliminate unwanted fungi and compounds which retard growth and thus restrict their diameter. The latter group will be discussed in the next section.

Of the selective compounds or media that could be found in the literature

most were for fungi not being important for seed health testing or at least not for the infections available to the author. An exception were those reported for species of *Fusarium*. Quite a number of media have been claimed as being suited for their selective isolation from soil. These have recently been evaluated by PAPAVIZAS (1967). The present author has tried three media in preliminary tests, namely that of DENIS *et al.* (1966), containing sodium azide; that of GARCIA-ACHA *et al.* (1966), containing sodium propionate in Czapek-Dox agar; and the medium of NASH & SNYDER (1962), containing PCNB (pentachloronitrobenzene) in peptone agar. The first two media proved to be less suitable. The sodium propionate medium is supposed to be specific for *F. culmorum*. It was found that now and then a rapidly growing *F. culmorum* colony arose and no other species. Unfortunately this pathogen was infrequent in our samples and no definite conclusions could be drawn.

Further work was restricted to peptone-PCNB agar. This medium had many advantages, such as a simple composition: 1.5% Difco peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_20$, 50 p.p.m. terramycin (NASH & SNYDER used 300 p.p.m. streptomycin), 1000 p.p.m. PCNB (75% wettable powder), and 2% agar. It does not require to be sterilized, neither do the petri-dishes, provided they have been well cleaned with warm water.

The first point that needed attention was a study of the appearance of colonies that arose from the wheat seeds on this medium, an incubation of 7 days at 20°C in darkness being applied. On or close to the seeds various fungi succeeded in producing a small cushion-like area immediately around or underneath the seed but never produced a colony on the agar. This was only the case with *Fusarium* spp. It was found that *Mucor* spp. and *Botrytis cinerea* were able to give colonies on the medium as well but they could be easily identified by their scanty thin growth and remained restricted to an area around a single seed. This latter advantage applies also to the *Fusarium* colonies themselves. It was found that *Cephalosporium roseum* can also grow on this medium. Such colonies were found once when plating maize seeds on it. They were immediately recognizable by their pale-pink powdery appearance.

The next point was the possibility of distinction between the various *Fusarium* spp. After 7 days of incubation *Fusarium* colonies rarely sporulated, neither in darkness nor in NUV light, but after prolonged incubation they sometimes did. The form of spores produced on peptone-PCNB agar is different from that on PDA; they are swollen and distorted. Correct identification is difficult. As there was a difference between the colonies present, these were put into groups and from each of them transfers were made to PDA. In this way it was found

to be easy to distinguish *F. nivale* from the other *Fusarium* spp. *F. nivale* can be recognized at once by its larger flat regular colonies without aerial mycelium. They are pale salmon in colour, but sometimes colourless or white. The other *Fusarium* spp. were characterized by the production of abundant coarse white mycelium, sporulation being rare except for *F. avenaceum*. In NUV light this species appeared to produce concentric circles of orange spore masses. Unfortunately it had largely disappeared from the 1966 samples at the time of these experiments (originally it was frequent in them), whereas it was rare in the samples of 1967 (probably due to the warm dry summer). Its place in the latter samples was taken by *F. poae*. The colony appearance of *F. poae* is intermediate between that of *F. nivale* and the other *Fusarium* spp. They are less white than those of the other *Fusarium* spp. (without *F. nivale)*, the aerial mycelium is less dense or rather scanty and not coarse but delicate. It can always be distinguished by its semolina appearance caused by the microconidial sporemasses observable to the naked eye or with a magnifying glass. The base of the colony is similar to that of *F. nivale* but the edge is less regular. The typical odour is less pronounced than on PDA, but often still noticeable (Plate IV, Fig. 1 and Fig. 2).

Different *Fusarium* spp. sometimes grew from a single seed and then the flat growth of *F. nivale* could be detected underneath the aerial mycelium of other species. This could be noticed by holding the dishes against the light. By testing other seeds on this medium it was found that it was also good for the detection of *Fusarium* spp. in them, but the general colony appearance was somewhat aberrant. The same was true for colonies arising from inoculations of pure cultures on the medium. This can probably be explained by the fact that different types of seed provide a different nutritional addition to that supplied by the medium.

Table 81 gives the results obtained for a number of cereal samples by this new method and on PDA and blotters. One of the most striking facts is the enormously increased percentage of *Fusarium* spp. as compared with all other tests. This proves the important role played by interfungal antagonism in ordinary tests on e.g. PDA with terramycin. On the other hand the figures were of such high levels that they could hardly be considered agriculturally valuable so that it was felt necessary to reduce them to more significant proportions (cf. ch. 6).

For that reason NaClO pretreatment was used which gave results close to those obtained on PDA after NaClO pretreatment, as can be noted in the above tables. This again constitutes a proof for the pathogen reducing action of NaClO treatment.

Table 81. Comparison of average percentages of *Fusarium* spp. found on the selective peptone-PCNB agar and on PDA for cereal seed samples with and without NaClO pretreatment. Routine *Fusarium* blotter test results are also given.

seed	*Fusarium* spp.	number of samples	peptone-PCNB agar		PDA		blotters
			without NaClO	with NaClO	without NaClO	with NaClO	without NaClO
wheat	*F. nivale*	6	1.8	2.0	0.8	1.7	–
	other *Fusarium* spp.		28.3	14.3	11.4	6.8	–
	total *Fusarium*		30.1	16.3	12.2	8.5	18.8
rye	*F. nivale*	6	4.2	4.0	2.4	3.0	–
	other *Fusarium* spp.		13.0	5.0	4.5	2.3	–
	total *Fusarium*		17.2	8.0	6.9	5.3	4.4
barley	*F. nivale*	6	3.7	5.3	3.5	5.0	–
	other *Fusarium* spp.		27.5	2.8	3.2	2.0	–
	total *Fusarium*		31.2	8.1	6.7	7.0	7.5
oats	*F. nivale*	4	1.0	1.0	0.5	0.5	–
	other *Fusarium* spp.		65.5	11.8	9.0	11.0	–
	total *Fusarium*		66.5	12.8	9.5	11.5	4.8

Table 82. Effect of different NaClO pretreatments on *Fusarium* percentages found for 2 wheat seed samples on peptone-PCNB agar

NaClO pretreatment	sample A		sample B	
	F. nivale	*Fusarium* spp.	*F. nivale*	*Fusarium* spp.
none	7	84	26	14
10 minutes ½%	17	45	21	6
10 minutes 1%	24	17	19	2
10 minutes 2%	19	9	12	0
10 minutes 4%	12	5	15	0

Table 82 gives the results of an experiment on the effect of various concentrations of NaClO on the *Fusarium* levels found on peptone-PCNB agar. In the first sample (A), the *F. nivale* percentage was highest after the 1% NaClO pretreatment, due to the high percentage of other *Fusarium* spp. The real *F. nivale* percentage will be higher than 24% as the 1% NaClO can be assumed to have reduced it. This is in agreement with the figures obtained for sample B. Sample A is the same as that of table 32 (section 3.18).

The peptone-PCNB method is very easy and convenient. It requires no sterilization, only simple incubation conditions and permits fast and easy inspection. There is hardly any difference in results between incubation in the dark and in

Table 83. Effect of light conditions on the average percentage of *Fusarium* obtained on peptone-PCNB agar for 7 wheat seed samples (7 days at 20C° after NaClO pretreatment)

light conditions	*F. nivale*	other *Fusarium* spp.
7 days darkness	12.3	51.3
4 days darkness + 3 days NUV	14.7	50.9
7 days NUV	11.9	45.7
7 days daylight (room temperature ± 20 °C)	12.4	47.2

light (table 83). Sporulation is somewhat increased by prolonged incubation in NUV light but the percentages do not change; neither does colony appearance. The best incubation regime was found to be 7 days at 20 °C. After NaClO pretreatment this resulted in percentages close to those obtained on PDA. Prolonged incubation, however, often tends to yield higher percentages (table 84). The use of lower temperatures offers no advantages (same table) but requires a longer incubation time. This relation between temperature and time makes clear that in this method the temperature should be kept constant for a good standardization.

The effect of selective compounds in other methods was also examined. This was done in the perspex-freezing method, described in section 4.12, which allowed for a convenient and economic way of testing chemicals for selective effects. It was always necessary to add these compounds in combination with

Table 84. Effect of test duration on the percentage of *Fusarium* spp. obtained from a wheat seed sample on peptone-PCNB agar

incubation regime	NaClO pretreatment	*F. nivale*	other *Fusarium* spp.
7 days 20 °C	–	39	11
	+	28	6
10 days 20 °C	–	45	16½
	+	36½	9½
7 days 15 °C	–	31	11
	+	27	4½
14 days 15 °C	–	49	13½
	+	37½	7
14 days 10 °C	–	36½	10½
	+	31½	4

some antibacterial antibiotic (200 p.p.m. terramycin routinely being used). If this is omitted the perspex tests are spoiled by bacterial development.

Several chemicals were tested but only PCNB in concentrations of 500 or 1000 p.p.m. offered some prospects for *Fusarium* spp. (tables 85 and 86). The percentage *Fusarium* remained more or less the same, whereas that for other fungi was much reduced. *Mucor* species were not eradicated but rendered less troublesome by inhibited growth. This facilitated recognition of *Fusarium* spp. On the other hand their colony appearance is altered making it difficult to distinguish between the species. This does not only apply to mycelial growth but also to the spores, the shape of which becomes abnormal, rendering microscopic identification less reliable. This difficulty of species differentiation made the perspex plate method with PCNB less attractive.

When seeds were shaken with PCNB and then plated on PDA the percentage *Fusarium* found was also much increased (table 87). In blotter tests with germinating seeds PCNB treatment of the seeds as well as addition of PCNB to

TABLE 85. Effect of PCNB on the average percentages of some pathogenic fungi found by the perspex-freezing method in 10 wheat seed samples (7 days 10°C followed by freezing and then 7 days 20° + NUV)

pathogen	0 p.p.m.	500 p.p.m.	1000 p.p.m.
Fusarium nivale	15.4	16.3	15.9
F. graminearum + *F. culmorum*	6.2	6.7	6.6
F. avenaceum	5.1	5.4	6.9
Septoria nodorum	29.3	13.2	11.4
Helminthosporium spp.	0.9	0.0	0.0

TABLE 86. Effect of PCNB on the average percentages of some pathogenic fungi found by the perspex-freezing method in 5 oat and 6 barley seed samples (7 days 10°C, followed by freezing and then 7 days 20°C + NUV)

seeds	pathogen	0 p.p.m.	1000 p.p.m.
oats	*F. nivale*	4.2	4.9
	other *Fusarium* spp.	39.1	41.7
	Helminthosporium avenae	34.2	5.0
	H. sativum	0.7	0.0
barley	*F. nivale*	2.3	2.0
	other *Fusarium* spp.	13.1	15.7
	Helminthosporium spp.	27.0	3.8
	H. sativum	4.4	0.3

TABLE 87. Effect of PCNB and NaClO pretreatment of wheat seeds on the average percentages found for some fungi in subsequent agar tests with 10 samples

fungi	control	with NaClO pretreatment	seeds shaken with PCNB
Fusarium nivale	12.1	12.4	25.9
F. graminearum + *F. culmorum*	3.0	2.2	6.4
F. avenaceum	13.2	7.1	38.4
Septoria nodorum	27.0	25.7	4.1*
Epicoccum	33.7	8.4	23.2*
Alternaria	27.3	18.7	1.4
Mucor	10.0	0	0

* tiny colonies

TABLE 88. Effect of PCNB on the average results of the routine blotter test for *Fusarium* spp. with 10 wheat seed samples

blotter test symptoms	control	seeds shaken with PNCB	blotters wetted with 1000 p.p.m. PCNB
dead seeds + *Fusarium*	1.3	0.9	1.2
severe rootrot	3.8	0.6	1.1
light rootrot	24.4	6.1	8.1
total *Fusarium*	29.5	7.6	10.4

the blotters itself led to a decreased root rot percentage being observed, indicating that the PCNB destroys the pathogenic ability of the fungi (table 88).

In silversand and soil only a small scale experiment was performed with one wheat sample containing 57% *F. nivale* and no *Septoria nodorum*. It was sown in sterile silversand and non-sterile clay loam soil in 'tents' at 7 °C and 15 °C, 200 seeds being used per treatment.

Table 89 shows that in silversand PCNB reduces disease whereas in soil there is no effect at all visible. Apparently the physiology of *Fusarium* is altered in such a way, that not only the morphology of the fusaria, but also their parasitic capacity is affected. The viability and capacity for saprophytic growth are not affected, which together with the absence of competition explains the high percentages found on peptone-PCNB agar and in PCNB-treated seeds on PDA. The freezing method is intermediate here, as during the first 7 days of incubation the parasitic capacity is stressed and later on the saprophytic.

TABLE 89. Effect of shaking wheat seeds with PCNB on the percentage emergence and diseased seedlings after sowing in silversand and soil in 'tents'

medium	temperature and length of incubation	PCNB	%emergence	%disease
silversand	28 days at 7°C	–	76	21
		+	97	19
	12 days at 15°C	–	95	36
		+	98	12
soil	28 days at 7°C	–	74	27
		+	79	31
	12 days at 15°C	–	97	4
		+	95	4

5.7 EXPERIMENTS WITH GROWTH RETARDANTS

Growth-retarding agents are frequently used for isolation of soil organisms in order to allow better quantitative determinations by restricting colony diameters. Without them fast-growing fungi may cover the plate and thereby suppress or mask the slower growers. Another advantage is the more definite outline of the colonies which makes for an easier distinction. One of the first compounds being used for this purpose was Rose Bengal (MARTIN, 1950), which at the same time possesses bacteriostatic properties (SMITH & DAWSON, 1944). Others are oxgall (MILLER *et al.*, 1951; LITMAN, 1947) and various bile salts such as sodium taurocholate and sodium desoxycholate, which are probably the active constituents of oxgall. They are surface-active chemicals, and other such agents, e.g. household detergents, have also been tried (STEINER & WATSON, 1964, 1965).

The development of a satisfactory method for the determination of *Fusarium* spp. in cereals made the lack of a similar method for *Septoria nodorum* in wheat more seriously felt. No selective chemicals or treatments are known as yet for this latter fungus. As it is a slow grower and is easily outgrown by other fungi, notably *Epicoccum* (cp. section 5.8), the use of growth restricting agents might offer some prospects. Experiments were carried out in the perspex-freezing method and in agar with 50 and 100p.p.m. Rose Bengal; 10, 20 and 40p.p.m. sodium desoxycholate; 1000, 2000 and 4000 p.p.m. dehydrated oxgall, and 500 p.p.m. household detergent. All these agents, with exception of the detergent, facilitated the observation of *Septoria nodorum* on PDA. Oxgall was the most satisfactory, as Rose Bengal imparted a red colour to the colonies and sodium

desoxycholate had a greater effect on sporulation and morphology of various fungi. Although these disadvantages did not apply to the *Septoria* colonies themselves, a more normal appearance of the others as long as they remain present is preferable. In the perspex-freezing method the use of these chemicals changed the appearance and recognizability of the colonies too much and the percentages were lower than those of the controls and more difficult to obtain. Consequently, attention was focussed on the use of oxgall in PDA.

TABLE 90. Effect of different oxgall concentrations in PDA on the average percentages found for *Septoria nodorum* in 6 wheat seed samples after NaClO pretreatment

p.p.m. oxgall	*S. nodorum*
0	33.7
1000	35.3
2000	36.0
4000	34.3

The average percentages of *S. nodorum* obtained on PDA with 3 different oxgall concentrations did not differ much but 2000 p.p.m. seemed to be the best one (table 90). At this concentration *S. nodorum* always produced pycnidia and frequently also some characteristic curly white mycelium. The pycnidia usually sporulated, the petri-dishes being illuminated for the last 3 days with NUV light (after 4 days in darkness) at 20 °C. At 4000 p.p.m. the *Septoria* colonies became too tiny and only pycnidia were observed and no mycelium. At 2000 p.p.m. most fungi were very much restricted in growth and interference was less than at 1000 p.p.m.

The above results were obtained with seeds pretreated with NaClO in order to get a better impression of the effect on the fungus itself. Table 91 shows the

TABLE 91. Effect of 2000 p.p.m. oxgall on the average percentage of *Septoria nodorum* found on PDA for 11 wheat seed samples

oxgall	NaClO pretreatment	*S. nodorum*
–	+	16.1
–	–	21.0
+	–	26.0

data for 11 wheat samples non-pretreated on oxgall-PDA as compared with ordinary PDA with and without NaClO pretreatment. This enables a better evaluation of the method. It can be clearly seen that by the use of oxgall surface disinfection is less necessary and allows for higher *S. nodorum* percentages being found. The percentage of other fungi remained constant but their growth was restricted so that no problems with *Mucor* or *Botrytis* were encountered. *Septoria* appeared to be easily masked by *Epicoccum* (cf. section 5.8). On PDA with 2000 p.p.m. oxgall the *S. nodorum* colonies could be easily seen inside *Epicoccum* colonies as a clear area in which pycnidia could be observed by holding the plates against the light (Plate V, Fig. 1.)

5.8 Some experiments with artificial inoculation

The presence of interfungal antagonism in seed health tests recalls the question that was asked in connection with bacterial antagonism in chapter 3, viz. the role it would play in soil. In this connection we have to distinguish between masking (observation being hampered or prevented) and competition. Only the latter phenomenon is related to sowing value. Unfortunately it is hard to distinguish the former from the latter.

In tests of cereal seeds two pathogens that are nearly always present in rather high percentages are *Alternaria tenuis* and *Epicoccum nigrum*. These fungi undoubtedly have a masking effect in e.g. the agar test. It was not known, however, whether they would have any protective effect as well.

In order to obtain some insight into the latter possibility a wheat seed sample containing a high level of *Fusarium nivale* and low percentages of *Alternaria* and *Epicoccum* was inoculated with the latter two fungi. This was done by shaking the seeds in sporulating petri-dish cultures on PDA, which covered the seeds with spores and mycelium. They were then sown in sterile silversand and in non-sterile clay loam soil in 'tents' and kept at 7 °C. Table 92 gives the results which show a clear-cut reduction by the inoculation treatment of disease development in silversand, but not in soil. In the latter the soil-borne antagonism was apparently more important.

The opposite kind of experiment was also carried out, viz. the artificial inoculation of samples with *Fusarium nivale*. For this purpose two samples, A and B, were taken. Sample A was 1½ year old and contained hardly any fungi, whereas sample B was ½ year old and contained much *Alternaria* and *Epicoccum*. The samples were each divided into two parts, one half being pretreated with hypochlorite solution (1 % for 10 minutes) and the other half left untreated. The

TABLE 92. Effect of artificial inoculation of wheat seeds with *Epicoccum nigrum* and *Alternaria tenuis* on the percentages of emergence and disease in silversand (sterile) and non-sterile clay loam soil in 'tents' at 7 °C

treatment	silversand		clay loam soil	
	emergence	disease	emergence	disease
control*	70½	28	77¼	12¾
inoculated with *A. tenuis*	78	16¾	75½	14¾
inoculated with *E. nigrum*	76½	12¼	77	15¼

* the sample used for inoculation gave on PDA:

NaClO	*F. nivale*	*F. graminearum*	*F. avenaceum*	*Septoria nodorum*	*E. nigrum*	*A. tenuis*
–	57	3	8	0	17	30
+	59	1	5	0	8	23

seeds were then inoculated with *F. nivale* by mixing them with a spore suspension containing 5×10^5 spores per ml. The results are presented in table 93.

As sample B gave a much lower disease development than A, these data support that of the previous experiment. The hypochlorite pretreatment of sample B resulted in a somewhat greater disease development in silversand, but not in soil. Here the soil-borne antagonism was probably more important.

These experiments were carried out only once, with 400 seeds per treatment. Inoculation of wheat seeds with *Alternaria* and *Epicoccum* before sowing them in silty clay loam in the greenhouse at 15 °C did not result in any difference in disease development by *Septoria nodorum*.

It may be concluded that in wheat seed the two most common saprophytes have a limited protective value. Only under special circumstances, such as sterile conditions, can they reduce the effect of a pathogen like *F. nivale*. The soil flora seems to provide at least the same protection as do seed-borne *Alternaria* and *Epicoccum* in sterile silversand. It is likely that the percentage reduction is obtained by the elimination of infections with a low inoculum potential (next chapter).

It may be interesting to add that CSUTI *et al.* (1967) claimed that artificial inoculation of wheat seed with *Epicoccum nigrum* and *Alternaria tenuissima* reduced disease development by *Helminthosporium sativum*.

The limited protective value of *Alternaria* and *Epicoccum* against disease

TABLE 93. Effect of artificial inoculation with *Fusarium nivale* on the percentage of emergence and disease of 2 wheat seed samples in silversand and soil in 'tents' at 7 °C

sample*	NaClO pretreatment	inoculation	silversand		clay loam soil	
			emergence	disease	emergence	disease
A	–	–	98	0	87½	½
	–	+	64½	36	71¼	23
	+	+	69½	34½	69	17½
B	–	–	94¾	4¼	96	1¼
	–	+	91	5¼	94¼	3¼
	+	+	94¼	12¾	90	1½

* the two samples used gave on PDA:

sample	NaClO pretreatment	*F. nivale*	*F. graminearum* + *F. culmorum*	*Septoria nodorum*	*Epicoccum*	*Alternaria*
A	–	0	0	0	2	5
	+	0	0	0	1	0
B	–	1	4	2	73	56
	+	3	2	5	40	49

development from seed-borne *Septoria nodorum* was also shown by the insignificantly low correlation between the percentages of the first two fungi in 74 wheat seed samples on PDA, and disease symptoms caused by *S. nodorum* in greenhouse tests.

The correlation between percentage *S. nodorum* on PDA and disease symptoms in the greenhouse was + 0.85 and is highly significant. Negative correlations were found between the percentages for *Septoria* and *Epicoccum*, viz. of –0.47 and –0.44 on PDA without and after hypochlorite pretreatment, respectively. These figures are in good agreement with the observation that *Septoria* was often found to be masked by *Epicoccum*. Very small *Septoria* colonies often were surrounded entirely by *Epicoccum* and thereby prevented from further growth. Only scrutinous examination of the plates brought such colonies to light. The use of growth-restricting agents such as oxgall (section 5.7) reduced this masking effect.

5.9 Discussion

It appears from survey work that the flora of seeds can be very extensive. Machacek *et al.* (1951) isolated 102 species of organisms belonging to 43 genera from seeds of wheat, oats and barley. Pepper & Kiesling (1963), published a list gathered from the literature of about 300 seed-borne organisms claimed to have been isolated from barley seeds. Malone & Muskett (1964), described 77 seed-borne fungi isolated by the authors mainly from oat and flax seeds.

In incubation tests many of these fungi can be stimulated to grow and interfere with pathogens. In this publication we have designated all interference with the expression of pathogens in seed health tests as antagonism.

It will have become clear that surface disinfection or removal of superficial 'seed' structures insufficiently discriminates between pathogens and saprophytes and may result in too low pathogen percentages found.

The use of different temperatures as was advocated by Neergaard & Saad (1962) for rice seed does not offer a good solution either, at least not for agar tests. An illustration of this was provided by *Fusarium nivale* of which the optimum temperature for growth on PDA was found to be around 20°C. At lower temperatures it was perhaps in a better competitive position, but no significantly higher percentages were obtained. The easier recognition (NaClO pretreatment still needed) was offset by the longer incubation period required.

The growth of *Septoria nodorum* on PDA is very much retarded by lower temperatures, being already quite slow at 20°C. Brönnimann (1968), however, claims that incubation of wheat seeds after hypochlorite pretreatment, for 7 days on PDA (Difco) under continuous illumination with 18000 lux gave very good results. The colonies were apparently of the same type as we obtained on PDA with oxgall (xection 5.7), viz. containing many sporulating pycnidia, but little aerial mycelium. It is stated, however, that due to technical difficulties this method will be less suited for routine application.

It will be clear that only the use of more selective methods can ameliorate this situation and allow for more absolute figures. Unfortunately only a few are right now available, but with the continued appearance of new selective chemicals reported in the literature their number may rapidly increase. Those given in this chapter can serve as a begin. The use of selective methods, notably that with peptone-PCNB agar for *Fusarium* spp., dramatically illustrates the importance of the factor antagonism. Antagonism also allowed for the explanation of the fact that soaking of seeds in terramycin solutions (ch. 3), sometimes results in lower pathogen percentages found due to copious development of many other fungi besides the pathogens.

During the stay of the author at the Wageningen Station the same phenomenon of mercury resistance was observed in *Helminthosporium avenae* as has been reported from Scotland (NOBLE *et al.*, 1966) and from Northern Ireland (MALONE, 1968), in the latter case only for a part of the strains (samples). When such mercury-treated seeds were plated on agar they yielded considerably higher percentages than untreated ones, again indicating the importance of the factor antagonism.

It is a well-known phenomenon that seed-borne fungi tend to decrease during storage. There are some cases, however, such as *Helminthosporium avenae* in oat and *Septoria nodorum* in wheat seed, which are very long lived. When their fungal competitors decline they get in a better position which offers an explanation for the fact that seed samples containing them often yield higher percentages with increasing age of the samples (MALONE, 1962 and DE TEMPE, pers. comm., for *H. avenae* and BRÖNNIMANN, 1968, for *S. nodorum*, as well as personal observations on both infections).

The question can be asked whether the saprophytes present do not interfere with disease development in soil, so that it would be better to have them incalculated in the results. Here the problem presents itself of distinguishing between masking and inhibiting effects. Masking bears no relation to what may happen in soil, whereas also different environmental factors give another pathogen/saprophyte balance. It is therefore better to eliminate the factor antagonism altogether. The problem called forward by this attitude will be dealt with in the next chapter.

6 INOCULUM POTENTIAL

6.1 INTRODUCTION

The results of incubation tests for seed health are stated in terms of percentage of seeds harbouring a particular pathogen. Such a statement tells little about the severity of the infection. In the preceding chapters we have seen the development of techniques and methods enabling us to find ever increasing percentages by the elimination of the factors antagonism and host resistance.

These factors do undoubtedly play an important role in the difficulty experienced in the standardization of incubation methods. They constitute a source of variation and render inspection more difficult, thereby also introducing errors

of personal judgment. But the high percentages often obtained after their elimination call forth the question of their meaning.

An approach to the problem could sometimes be a distinction between severe and light infections. An example thereof is the division into 3 groups of the infection percentages as determined by the routine blotter method for *Fusarium* spp. The group of non-germinated seeds covered by *Fusarium* mycelium is probably unimportant for practical purposes, as it is eliminated in germination figures. Originally this group was called: killed seeds, but this term had to be withdrawn, because it became clear from a comparison between blotter tests with and without NaClO pretreatment, that the disinfection decreased this group with the same amount as the group of non-germinating (dead) seeds increased (cf. table 69). The infections eliminated by the NaClO pretreatment are consequently not deep-seated but are probably secundary.

The other two groups are those of the severely and lightly infected seedlings, in which the symptoms of brown root rot are respectively accompanied or not by root abnormality. It was thought that this would constitute a demonstrative example of such a grouping into more or less severe infection. The correlations givenand discussed in section 5.2 in connection with the influence of temperature did show, however, that the ratio between light and severe infections can be shifted easily.

Another difficulty with the distinction between light and severe symptoms is the fact that the former may be worse than the latter. Severe infections may cause pre-emergence death and affect only the germination figure. Light infections can give rise to a more delayed disease development and serve as later foci of infection in the field (e.g. *Ascochyta pisi* in peas).

On the other hand infections may be so light that they do not stand a chance in natural soil. It can be assumed that this is the case with the *Fusarium* that disappears after normal NaClO pretreatment in tests on peptone-PCNB agar. Another example is that supplied by SCHERMER & SCHIPPERS (1965), SCHIPPERS & SCHERMER (1966) for *Verticillium albo-atrum*-infected *Senecio vulgare* seeds, which only gave disease development under sterile conditions. The following reasoning can be used. The severity of the infection depends on the quantity of viable units present on and in the seed. These units can take the form of various propagules, but usually that of mycelium. Some method of quantitating these units might give an idea of the infection severity, but unfortunately this is very hard to do for mycelium.

A number of different approaches was tried, one of them being the determination of the number of spores by the shaking method after incubation in a blotter

test, the seedling being cut off and the seed shaken with alcohol. The number of spores was then divided by the infection percentage to provide a so-called infection index. The method was rather laborious and the shakings contained much dirt (*Alternaria*, starch, etc.). Worse was that the results were not reproducible for the same sample, pointing out that there were too many variables affecting sporulation. Moreover the method would have the inherent error of differences between sporulating abilities of various strains of the pathogen.

Another possiblity for estimating the severity of seed-borne infection is the use of escalated surface pretreatments. It would involve the use of the maximal method for an infection combined with surface treatments of increasing severity. The results could then be compared with disease development in soil and the pretreatment giving the best agreement chosen for routine application.

6.2 NaClO PRETREATMENT AND INOCULUM POTENTIAL

The preceding section introduced a new factor, viz. that of inoculum potential of the seed-borne pathogen. The term will be used here according to the definition of GARRETT (1956), who defined it as follows: "inoculum potential may be defined as the energy of growth of a parasite available for infection of a host at the surface of the host organ to be infected." In section 5.3 we saw that NaClO pretreatment reduces the percentage of infection found, but that this is less noticeable on agar media than on blotters and in soil.

The following explanation can be given. Suppose we have a seed carrying a saprophyte and a pathogen, a unit of which we indicate as S and P respectively, the seed carrying 25 S and 10 P. We assume, moreover, that the NaClO pretreatment removes a superficial flora consisting of 20 S and 5 P and leaves a more deeply seated flora consisting of 5 S and 5 P. This would mean that the ratio of S:P had been changed from 5:2 to 1:1. The number of propagules of the pathogen has been reduced from 10 to 5. In the saprophytic agar method the 5 units of the pathogen are still sufficient, especially as competition is reduced by 60%. In the blotter method, however, 10 units provide a sufficient inoculum potential for infection but 5 propagules may be below the treshold level required. This treshold level can be changed by altering the host vigour (ch. 4). As the host vigour in soil may differ from that in blotter tests, the treshold may be different too.

An idea of the effect of increasing the severity of the surface pretreatment with NaClO in various media can be had from table 94 (cf. also table 82 for peptone-PCNB agar). These figures underline the above reasoning on inoculum poten-

TABLE 94. Effect of NaClO pretreatment of increasing severity on the restults obtained with 4 different methods for one wheat seed sample

method*	sings or symptoms	pretreat-ment**	duration of pretreatment in minutes				untreated control
			5	10	20	40	
A	*Fusarium* spp.	a	17	18	21	14	20
		b	11	12	10	13	
		c	16	11	13	10	
		d	12	6	6	2	
	Septoria nodorum	a	68	59	52	59	81
		b	56	64	63	55	
		c	57	53	43	29	
		d	50	52	42	40	
B	total *Fusarium*	a	26	17	14	15	40
		b	15	9	10	7	
		c	11	8	8	5	
		d	4	4	1	3	
C	*S. nodorum*	a	26	26	21	-	82
		b	-	13	17	-	
		c	-	5	4	-	
		d	-	4	0	-	
D	disease (coleoptyle lesions)	a	50	41	49	43	68
		b	38	37	39	30	
		c	26	29	20	25	
		d	16	17	12	14	
	emergence	a	93	94	94	93	73
		b	88	91	92	87	
		c	86	80	81	75	
		d	95	94	89	87	

* A = agar test on PDA
B = routine *Fusarium* blotter test
C = routine *Septoria* blotter test (The wheat sample used (Tadorna) was a good protuberance producer)
D = greenhouse test in silty clay loam at 15 °C

** a = ½% NaClO
b = 1% NaClO
c = 2% NaClO
d = 30 seconds alcohol dip followed by 1% NaClO

tial. It was found that variations in NaClO-concentration are more important than duration of the treatment. It is also clear that escalation of NaClO pretreatment only makes sense for such selective methods as for instance the use of peptone-PCNB agar as only here the effect of escalation will be clearly visible. On PDA the change in pathogen/saprophyte relation interferes, whereas in blotters the inoculum potential itself is affected.

The *Septoria nodorum* infection of wheat seed served as a good illustration for the importance of maximum methods. The disease percentage found in soil (greenhouse test at 15°C) was always higher than that obtained on agar (section 6.3 and table 95). The Kietreiber blotter method gives a better agreement, but only for those wheat varieties that produce the characteristic wart symptom, which most Dutch wheat varieties for instance, do not show. The ordinary agar method is usually carried out after surface pretreatment which reduces the infection percentage as we saw in section 5.3 and 5.7.It is therefore clear that the oxgall method which requires no surface disinfection (cf. section 5.7) is to be preferred.

The quite different effect of PCNB in the saprophytic and parasitic methods (cf. section 5.6), can also be explained in terms of inoculum potential (sensu GARRETT, 1956). Although the number of pathogen units remains unchanged, the PCNB certainly affects the energy of growth of the pathogen available for infection.

6.3 THE USE OF THE CHRISTENSEN METHOD

CHRISTENSEN (1957) has described a special method for the determination of storage fungi in cereals. One hundred seeds are ground in 0.2% water agar in a Waring Blendor, and then the suspension is poured into petri-dishes after being diluted 1:5000, resulting in the equivalent of $^1/_{50}$ seed per petri-dish. The number of colonies arising on the plates can be used as an index for the prevalence of the organisms in the sample.

For seed-borne pathogens this method would be less usefull because one severely infected seed with 100 units between 99 non-infected ones would give the same index as 100 seeds containing 10 units each. Although this argument can, to a certain extent, also be used against the following experiment, it was nevertheless performed to obtain a better insight into the matter of inoculum potential.

The *Septoria nodorum* infection of wheat was chosen for this experiment as its disease percentages in soil can be more easily determined, especially in sam-

ples containing relatively little or no *Fusarium*. A number of such samples happened to be available when this experiment was performed. In it the *Septoria* percentage on PDA, as well as the number of units found by the Christensen method, were determined for wheat seeds that had either been pretreated or not with 1% NaClO for 10 minutes. The data obtained were then compared with disease development from the same seeds sown in silty clay loam in the greenhouse kept at 15°C. For the Christensen method an Ultra Turrax apparatus (2 minutes at 24000 rpm) instead of a Waring Blendor was used.

TABLE 95. Effect of NaClO pretreatment on inoculum potential as found by 3 methods

sample	pretreatment	agar method (PDA)			Christensen method			greenhouse test at 15°C	
		Septoria nodorum	*F. nivale*	*Fusarium* spp.	*Septoria nodorum*		*Fusarium* spp.	emergence	disease
					1st det'n	2nd det'n			
1	none	77	1	3	424	*	0	91	80
	1% NaClO	60	4	1	8	60	0	98	48½
2	none	46	10	1	70	152	1	92½	61½
	1% NaClO	31	3	0	1	32	0	98	21
3	none	44	0	3	53	31	3	95½	82½
	1% NaClO	31	1	1	16	14	0	95	17½

* could not be counted due to abundant development of colonies of a *Phoma* sp.

The results obtained for 3 wheat seed samples are presented in table 95. Two *Septoria* counts are given for the Christensen method for each sample, the second one being obtained two weeks later. They have both been given to illustrate the poor reproducibility of the method for these infections. A difficulty with this method was also that sometimes large numbers of a *Phoma* sp. crowded the plates and made a correct count of *S. nodorum* impossible. Two other samples had to be discarded for this reason.

The data presented in the above table show that the agar percentages found for *S. nodorum* are on the low side when compared with disease development in soil, which is probably largely due to it being masked or prevented from growth by other fungi (cf. section 5.7). On the other hand the NaClO pretreatment reduces the disease percentage in soil more than the infection percentage found on agar (cf. section 6.2).

In the Christensen method the number of pathogen units found shows more or less the same trend as in soil. Hence the reduction in inoculum potential is better indicated by the Christensen than by the agar method. This suggests the possibility of developing a method by which disease percentages could be weighed.

A question that may bear some relation to the problem of inoculum potential is that of the importance of glume infections. In this connection the results of an experiment with barley seed containing *Helminthosporium gramineum* can be given.

In Dutch barley samples this fungus is very rare but we received some barley samples from the Finnish Seed Testing Station at Helsinki, which had been sown there in experimental fields. Table 96 gives the infection percentages found in these samples at Wageningen and the disease percentages found in Finland in the field. The weather records in Finland indicated conditions most likely to be favourable for the disease although strains of this pathogen may differ in optimum temperatures for disease development (ISENBECK, 1939).

TABLE 96. Infection levels of 4 Finnish barley samples with *Helminthosporium graminearum*

sample	variety	field disease in Finland	blotter method	freezing method	agar method (PDA) with 1% NaClO pretreatment
1	Pirkla	0.7	13	34	6
2	Otra	3.2	71	73	62
3	Otra	5.7	78	96	89
4	Otra	6.0	88	100	95

At Wageningen the seeds were sown in 'tents' in silversand at 7°C and inspected for stripe symptoms after 6 weeks when the plants were in the 3 leaf stage. The number of stripe symptoms was then recorded and all the apparently still healthy leaves were put on blotters moistened with 200 p.p.m. terramycin, frozen and then incubated at 20°C in NUV. The results are given in table 97.

It follows that the relation between laboratory figures, field and soil tests was very poor for this pathogen which is so highly adapted to seed transmission. The total infection percentage in the silversand test is higher than in soil. It may have been important in this case, that the samples were rather 'dirty' viz. containing many other fungi Antagonism may have played a role. If this is so, then it is quite interesting that such a well adapted pathogen is so sensitive, even under the favourable conditions provided.

R. KENNETH (pers. comm.), mentionned that *Helminthosporium teres* only

TABLE 97. Results of 'tent' experiments with the 4 Finnish barley samples of table 96

sample	% seedlings with stripe symptoms	% seedlings giving rise to *H. gramineum* in subsequent blotter test	total % seedlings carrying *H. gramineum*
1	0	3	3
2	1	7	8
3	2	10	12
4	2	9	11

succeeds in infecting seedlings if it has penetrated into the caryopsis, infections restricted to the hulls being of no consequence. Seeds from sample number 3 (table 96) were therefore dehulled and retested by the blotter method. This resulted in a drop from 96 to 57%. The dehulled sample was then tested in 'tents' in the same way as the hulled samples and gave a total of 14% infected seedlings.

The explanation suggesting itself is that although other factors are likely to be involved, the removal of the glumes eliminated many superficial infections (96–57 = 39%) incapable of causing infection, and at the same time many saprophytes.

That only 12 to 14% seemed capable of infecting seedlings is probably best explained by a combination of the factors antagonism and inoculum potential. Under the conditions provided the latter was perhaps below the infection threshold for all but 12 to 14% of the seeds.

In the case of the oat seeds of table 76 (section 5.4), on the other hand, the dehulling seems to have brought the total inoculum potential below the infection threshold in many infected seeds, thereby reducing the disease development in silversand.

6.5 Discussion

In this chapter a fourth factor influencing the outcome of incubation methods was mentioned, viz. that of inoculum potential (sensu GARRETT, 1956). In the discussion of the experimental results of previous chapters this same factor was also often involved.

HEALD (1921); HEALD & BOYLE (1923), studying seed-borne *Tilletia caries* in wheat, noted that a few hundred spores per seed are required for appreciable disease development in the field. COLHOUN (1964), found very high inoculum

levels to be required in case of artificial inoculation of seeds with spores of *Fusarium* spp. for causing disease development after sowing (cf. COLHOUN & PARK, 1964).

Various theories for the need of larger numbers of propagules (i.c. spores), have been discussed by GARRETT (1966).

A certain threshold level is required for infection. This level is not fixed but shifts with the conditions met. It is affected by the environment, the effect of which is again an indirect one, acting via the pathogen itself but mainly via host vigour and the seed (and soil) microflora. Changing of the host vigour to higher or lower levels will shift the infection threshold. It will also be shifted by improving conditions for antagonistic organisms. An example of the latter case is the wet blotter effect of chapter 3. Many examples can also be found in chapter 5 on interfungal antagonism. The higher the number of units of the pathogen, i.c. the inoculum potential, the more it will be in a position to overcome competition. This is a general finding in work on soil-borne pathogens as well.

The preceding discussion was somewhat simplified in that it purposely left out the factor of site of infection. This factor is complex in itself and can be split up into two categories, namely depth of penetration and localization in relation to the embryo. That superficial infections are more sensitive than deeper seated ones was observed on many occasions during this work. This was for instance already apparent in section 3.3 in connection with the reaction of artificial (= superficial) infection of seeds to the WBE. It was also observed in the previous section (6.4) in connection with the importance of glume infections.

The factor of depth of penetration is hard to separate from that of inoculum potential and for practical purposes such a separation can hardly be considered as necessary. An infection that has penetrated deeply into the tissues will in all likelyhood have a higher inoculum potential than one that has penetrated less deeply. Therefore surface pretreatments in selective methods can correct for amount of inoculum as well as depth of penetration.

The location of the infection on the seed will also bear a relation to inoculum potential, as the higher the latter, the greater the chances of it being present close to the embryo.

In this chapter we have tried to show that the use of highly selective methods is very attractive, but that some method to measure 'disease potential' is also required. The latter can perhaps be obtained by a combination of disease percentage and inoculum potential.

Possible approaches seem to be either the use of escalated surface pretreat-

ments or the development of a method giving some idea of the number of pathogen units per seed.

The first approach goes out from the idea that a certain severity of pretreatment will eliminate the more superficial infections and enable to measure the remaining ones. An example is a NaClO pretreatment preceding the peptone-PCNB method. It seems to be the simplest solution of the problem to indicate a justified method for evaluating *Fusarium* in cereal seeds.

An example of the second approach is the use of the Christensen method along with the ordinary test giving the percentage of infected seed. This would be more complicated and requires more additional work to achieve better standardization.

Unfortunately the author has only been able to carry out some preliminary investigations into this problem Further work will undoubtedly produce simple methods giving results of a greater practical applicability.

7 THE USE OF CORRELATION COEFFICIENTS

7.1 Introduction

As pointed out in chapter 1, the relation between laboratory results and field performance was considered very important by the Wageningen Station. It will be evident from the preceding chapters that such a relationship is difficult to established in quantitative terms. Seed health data based on incubation methods are given as percentage of infected seeds. But we have seen that the factor inoculum potential enters into the picture, as well as antagonism caused by seed-borne organisms, and also host vigour, not to mention race or strain of the pathogen and host variety. Climatic and edaphic factors will interact with the seed-borne factors to make for a completely unpredictable outcome. Moreover it is usually only possible to judge the effect of seed-borne infection in soil on emergence and sometimes also on the number of diseased seedlings, which does not present a complete picture of the importance of the infection (section 7.4).

When the present work started the intention was to study the relationship between seed health test figures and disease development in soil. Several factors influencing the results of the methods used were found which can be regarded as additional reasons for the low correlations obtained of old. The main attention was then shifted to a study of the causes of the variations in results, as discussed in preceding chapters.

A large correlation experiment had already been started by then. For this purpose the *Fusarium* and *Septoria* infections of wheat seed were chosen. Various methods and modifications of them were compared.

The reasons for this choice were in the first place that the above fungi can cause severe disease in the emergence stage.

Secondly, a wide range of samples more or less infected with these fungi is easily obtainable in the Netherlands.

In the third place, the methods of detecting them were far from satisfactory and, consequently, a comparative evaluation of the available methods was interesting.

7.2 Methods used and the figures obtained

The above correlation experiment was carried out with 74 Dutch wheat seed samples of the 1964 harvest and again with 136 such samples of 1965.

During the season 1964–1965 a number of methods was used and the results were studied. The next season the program was somewhat changed. Consequently a comparison of the results obtained with both series is only partly possible. For all methods 400 seeds per sample were used, except for the agar method in which we used 200 seeds. The methods are given in the following list. In it A, B, and AB indicate whether the method was used for the first, the second, or both series of samples.

- 1–AB The routine blotter test for Fusarium spp., viz. 3 days at 10 °C, followed by 3 days at 20 °C in darkness
- 2–B As 1, but with NaClO-pretreated seeds
- 3–A A modification of the above blotter test, having a temperature regime of 3 days at 10 °C, followed by 3 days at 15 °C
- 4–AB The routine blotter test for *Septoria nodorum* (Kietreiber method)
- 5–AB An agar test (on PDA), without NaClO pretreatment
- 6–AB An agar test (on PDA), after NaClO pretreatment
- 7–AB A greenhouse test in stilty clay loam at 15 °C, in which disease symptoms and emergence were noted
- 8–A As 7, but with NaClO-pretreated seeds
- 9–AB As 7, but with Ceresan-treated seeds
- 10–B A greenhouse test in steamed greenhouse soil at 7 °C, in which disease symptoms and emergence were noted
- 11–B An experiment in which the seeds were sown in jiffy pots (10 cm in diameter, 25 seeds per pot), which were dug in outdoors where they

experienced frost and snow immediately after sowing in spring. Only emergence was noted.

12–AB A field experiment in very heavy clay soil of the experimental plots of the Station. Only emergence was noted. (The results for the first series of samples were unreliable due to mice damage and had to be discarded. The second series experienced a spell of winter weather shortly after sowing in March).

13–A As 12, but with NaClO-pretreated seeds

14–AB As 12, but with Ceresan-treated seeds

TABLE 98. Average percentages obtained with test 1, 2 and 3 (blotter tests for *Fusarium*)

	category	1964—1965 test 1	1964—1965 test 3	1965—1966 test 1	1965—1966 test 2
a	*Fusarium* on dead seeds	1.57	1.64	2.84	1.62
b	severe symptoms	2.47	5.00	4.23	3.17
c	light symptoms	17.04	14.67	21.55	13.74
d	total *Fusarium*	21.08	21.32	28.62	18.53
e	epiblast browning	14.56	9.18	4.03	2.58
f	abnormal seedlings	2.85	4.26	5.42	6.20
g	non-germinated (dead) seeds	2.25	2.39	4.37	5.79

TABLE 99. Average percentages obtained with test 4 (blotter test for *Septoria*)

	category	1964—1965	1965—1966
a	seedlings with protuberances	12.89*	5.31*
b	seedlings with coleoptyle lesions or abnormal shoots without protuberances	6.29	27.77
c	seedlings with stripelets on coleoptyle	8.88	5.06
d	seedlings with brown stunted roots	2.73	4.98
e	root-symptoms and coleoptyle stripelets included in a and b	6.51	16.19
f	*Fusarium* on dead seeds	1.47	2.52
g	abnormal seedlings	2.20	0.84
h	non-germinated (dead) seeds	2.72	5.35
i	sum of a and b = % *S. nodorum*?	19.18	33.08
j	sum of c + d + e + f = % *Fusarium*?	19.59	28.75

* it should be taken into account that this figure is not produced equally by all cultivars, see text

15–B Official germination test in silversand in which germination capacity and coleoptyle browning were noted

16–B As 15, but seeds treated with Ceresan.

The average percentages of infection obtained by these tests are given in tables 98 to 102. In tables 103 to 108 a selection of some correlations computed between various test results is given.

Correlation coefficients for the first series of samples are significant on the 5 and 1% level above 0.23 and 0.33 respectively. For the second series the corresponding figures are 0.16 and 0.24.

TABLE 100. Average percentages obtained with test 5 and 6 (agar tests)

fungi	1964—1965		1965—1966	
	test 5	test 6	test 5	test 6
F. nivale	15.96	16.23	12.82	16.17
F. graminearum	7.93	4.54	3.79	1.79
F. culmorum	0.20	0.05	0.99	0.01
F. avenaceum	1.22	1.12	6.86	2.52
F. poae	0.62	0.74	0.34	0.40
Fusarium spp.	0.08	0.09	0.94	0.70
total *Fusarium*	26.02	22.77	25.04	21.59
S. nodorum	9.15	7.31	6.76	8.77
Epicoccum	44.65	25.22	65.09	25.93
Alternaria	47.45	42.99	46.80	37.93

TABLE 101. Average percentages obtained with test 7, 8, 9, 10, 11 and 13 (greenhouse and field experiments)

1964–1965	healthy seedlings	diseased seedlings	emergence
test 7	70.20	17.25	87.45
8	77.55	9.47	87.02
9	88.49	2.77	91.26
1965–1966			
test 7	68.77	12.44	81.21
9	86.33	0.40	86.73
10	46.06	21.52*	67.58
11	–		48.14
13	–		60.07

* of which 3.81 were stripe symptoms

TABLE 102. Average results obtained with test 15 and 16 for samples of 1965–66 (laboratory germination tests)

	test 15	test 16
germination capacity	84.7	89.3
coleoptyle lesions	38.5	8.2

TABLE 103. Correlation coefficients between the blotter test for *Fusarium* – at two temperature regimes – and the agar test with and without NaClO pretreatment, for the samples of 1964–1965.

blotter test	agar test						
	F. nivale		*F. graminearum*		total *Fusarium*	*Septoria nodorum*	
	with NaClO	without NaClO	with NaClO	without NaClO	with NaClO	with NaClO	without NaClO
3 days 10 °C and 3 days 20 °C							
Fusarium on							
dead seeds	−0.09	−0.03	+0.80	+0.69	+0.49	−0.18	−0.16
severe symptoms	+0.26	+0.25	+0.59	+0.59	+0.57	−0.08	−0.07
light symptoms	+0.67	+0.60	+0.13	+0.23	+0.57	+0.12	+0.10
epiblast browning	+0.18	+0.13	+0.02	+0.04	+0.17	+0.78	+0.77
3 days 10 °C and 3 days 15 °C							
Fusarium on							
dead seeds	−0.14	−0.05	+0.77	+0.67	+0.44	−0.08	−0.05
severe symptoms	+0.54	+0.50	+0.30	+0.32	+0.60	−0.04	−0.04
light symptoms	+0.64	+0.66	+0.15	+0.26	+0.62	+0.17	+0.15
epiblast browning	+0.15	+0.07	+0.05	+0.09	+0.18	+0.76	+0.75

TABLE 104. Correlation coefficients between the routine blotter test for *Fusarium* and the agar test (PDA), with and without NaClO pretreatment for the samples of 1965–1966; *I Fusarium* on dead seeds; II severe symptoms; III light symptoms; IV epiblast browning

blotter test	agar test							
	F. nivale		*F. graminearum*		total *Fusarium*		*S. nodorum*	
	with NaClO	without NaClO	with NaClO	without NaClO	with NaClO	without NaClO	with NaClO	without NaClO
without NaClO								
I.	+0.23	−0.15	+0.29	+0.45	+0.43	+0.44	−0.27	−0.28
II.	+0.62	+0.54	+0.08	+0.19	+0.58	+0.55	+0.24	+0.25
III.	+0.63	+0.69	+0.03	−0.04	+0.60	+0.59	−0.07	−0.08
IV.	−0.01	−0.01	−0.13	+0.10	+0.02	+0.19	+0.34	+0.23
with NaClO								
I.	+0.18	−0.22	+0.27	+0.31	+0.38	+0.29	−0.24	−0.26
II.	+0.53	+0.45	−0.02	+0.16	+0.50	+0.47	−0.20	+0.17
III.	+0.70	+0.78	−0.06	−0.09	+0.61	+0.54	−0.06	−0.07
IV	+0.40	+0.31	−0.03	+0.17	+0.41	+0.49	−0.08	−0.13

Table 105. Correlation coefficients between the *Fusarium* blotter test (at two temperature regimes) and the agar test (after NaClO pretreatment) on the one hand, and greenhouse test results (at 15 °C) on the other (74 wheat samples, 1964 harvest)

laboratory test		greenhouse test		
		healthy seedlings	diseased seedlings	emergence
PDA	*F. nivale*	+0.10	+0.04	+0.24
(after NaClO	*F. graminearum*	−0.15	−0.11	−0.43
pretreatment)	total *Fusarium*	+0.01	−0.07	−0.07
	S. nodorum	−0.73	+0.87	−0.19
blotter test	*Fusarium* on dead seeds	−0.17	−0.17	−0.56
(3 days 10 °C+	severe symptoms	−0.22	+0.01	−0.40
3 days 20 °C)	light symptoms	−0.02	+0.17	+0.20
	total *Fusarium*	−0.11	+0.11	−0.07
	epiblast browning	−0.50	+0.69	−0.01
	abnormal + dead	−0.18	−0.14	−0.52
blotter test	*Fusarium* on dead seeds	−0.24	−0.08	−0.56
(3 days 10 °C+	severe symptoms	−0.13	+0.11	−0.09
3 days 15 °C)	light symptoms	−0.03	+0.20	+0.22
	total *Fusarium*	−0.14	+0.18	−0.01
	epiblast browning	−0.47	+0.64	−0.22
	abnormal + dead	−0.18	−0.17	−0.57

TABLE 106. Correlation coefficients between laboratory test observations and emergence in soil tests for 136 samples of the 1965 harvest. I Agar test without pretreatment; II Agar test after NaClO treatment; III Blotter test without pretreatment; IV Blotter test after NaClO treatment; V Silversand test, untreated; VI Silversand test, Ceresan treated.

laboratory test	soil tests					
	greenhouse test			jiffy-pot exp.	field test	
	15°C untreated	15°C Ceresan treated	7°C untreated	untreated	untreated	Ceresan treated
I. *F. nivale*	−0.01	+0.10	−0.46	−0.46	−0.17	−0.12
F. graminearum *F. culmorum*	−0.29	−0.20	−0.09	−0.25	−0.22	−0.20
total *Fusarium*	−0.29	−0.13	−0.49	−0.59	−0.39	−0.37
S. nodorum	+0.33	+0.36	+0.12	+0.40	+0.29	−0.33
II. *F. nivale*	−0.29	−0.16	−0.55	−0.57	−0.30	−0.24
F. graminearum *F. culmorum*	−0.24	−0.19	−0.06	−0.27	−0.23	−0.20
total *Fusarium*	−0.27	−0.23	−0.58	−0.64	−0.42	−0.39
S. nodorum	+0.34	−0.36	+0.36	+0.42	+0.17	+0.22
III. dead seeds with *Fusarium*	−0.51	−0.53	−0.33	−0.47	−0.35	−0.25
severe symptoms	−0.18	−0.08	−0.60	−0.54	−0.26	−0.34
light symptoms	+0.03	−0.06	−0.57	−0.48	−0.26	−0.28
total *Fusarium*	−0.40	−0.36	−0.61	−0.50	−0.34	−0.26
epiblast browning	+0.07	+0.08	−0.06	+0.02	−0.12	+0.04
IV. dead seeds with *Fusarium*	−0.42	−0.41	−0.21	−0.32	−0.17	−0.20
severe symptoms	−0.16	−0.06	−0.44	−0.50	−0.27	−0.19
light symptoms	+0.08	+0.18	−0.46	−0.41	−0.20	−0.17
epiblast browning	−0.07	−0.01	−0.21	−0.20	−0.20	−0.19
V. germination percentage	+0.72	+0.80	+0.52	+0.66	+0.39	+0.43
disease percentage	+0.11	+0.17	−0.19	−0.09	−0.10	+0.03
VI. germination percentage	+0.66	+0.83	+0.35	+0.53	+0.46	+0.52

TABLE 107. Correlation coefficients between agar test results and coleoptyle lesions in greenhouse tests for the samples of 1965–1966

greenhouse test		agar test			
		F. nivale		*S. nodorum*	
		after NaClO	without NaClO	after NaClO	without NaClO
at 15°C					
untreated	lesions	−0.19	−0.02	+0.81	+0.77
Ceresan treated	lesions	+0.18	+0.08	−0.18	−0.19
at 7°C					
untreated	lesions	−0.04	−0.04	+0.29	−0.38
	tiny stripes	+0.29	+0.18	−0.30	−0.26
	other lesions	−0.15	−0.11	+0.43	+0.50
	lesions + abnormals	−0.04	−0.02	+0.59	+0.53

TABLE 108. Correlation coefficients between the *Septoria* blotter test (Kietreiber method) on the one hand and the greenhouse test (15°C) and agar test results on the other, for the samples of 1964—1965

		protuberances
greenhouse test	healthy seedlings	—0.73
	diseased seedlings	+0.80
	emergence	—0.27
PDA (after NaClO)	*Septoria nodorum*	+0.89

7.3 CONCLUSIONS DRAWN FROM RESULTS OF PREVIOUS SECTION

When studying the correlation data one has to take into account that high coefficients (which are rare) are more surprising than low ones. This is explained by the complex nature of the factors involved and the many sources of error, such as: sampling errors, errors of judgment, environmental factors, antagonism, presence of *Fusarium* and *Septoria* together, the complex character of the *Fusarium* infection, etc. It was in the first place important whether significant correlations were obtained. Differences between correlation coefficients were in addition taken to indicate possible trends that could be used as additional evidence or as a basis for future investigation. Correlation coefficients and no regressions were used as we are more interested in the proportionate importance than in the amount of change.

It may be best to start with a comparison between blotter and agar test results. The question to be resolved was the role of the various *Fusarium* species in the blotter test symptoms. In section 5.2 and tables 62 and 63 on the effect of temperature we already saw, that *F. graminearum* could be isolated from dead seeds with *Fusarium* mycelium and from seedlings with severe and light root rot symptoms; *F. nivale* only from the latter two. Changing the temperature regime to 3 days 10°C + 3 days 15°C did not alter the total amount of *F. graminearum* involved in the three groups, but caused symptoms induced by *F. nivale* to shift partly from light to severe.

It was also tried to carry out tests at still lower temperatures, but this had a number of disadvantages. In the first place the length of incubation needed to be increased and secondly the symptomatology did change. Some tests were carried out at 12°C for 10 days. The seedlings, however, had much shorter roots which caused difficulty in distinguishing the same groups. A large proportion was abnormal and there was also increased interference by *Septoria nodorum*. The *Fusarium* symptoms in the routine blotter method for *Septoria* (Kietreiber method) were used instead. This will be discussed later on.

If the results of the 1965 series are compared with those of the previous year it can be observed that the same picture emerges, provided that we take into account that the species composition of the *Fusarium* complex was somewhat different, *F. graminearum* being less prevalent especially after NaClO pretreatment. This may be the explanation for the low correlation shown with severe symptoms. As we add to this the observation made in chapter 5, that *F. nivale* may be negatively affected by the other *Fusarium* spp., it probably got an increased chance of getting involved in severe symptoms.

By plating roots on agar *F. avenaceum* was found to be of secundary importance as a cause of blotter test symptoms. The correlation between the two was also insignificant.

Artificial inoculation of wheat seeds was also used for trying to establish a relationship between *Fusarium* spp. and blotter test symptoms. Originally seeds were dipped in spore suspension but later on some mycelium from agar cultures freshly isolated from other wheat seeds was put into the seed raphe of *Fusarium*-free seeds. After inoculation the seeds were immediately blotter-tested in the normal way. Table 109 gives an idea of the kind of results obtained. It proved helpful to presoak the seed for about one hour in 100 p.p.m. terramycin solution.

For the first series of samples epiblast browning was strongly correlated with *Septoria nodorum*. Plating of brown epiblasts confirmed this by frequently yielding this pathogen. The former idea that epiblast browning could be regard-

TABLE 109. Effect of artificial inoculation with mycelium of *Fusarium* spp. into the raphe of wheat seeds on routine *Fusarium* blotter test results

	non-inoculated (control)	inoculated with			
		F. nivale	*F. graminearum*	*F. culmorum*	*F. avenaceum*
Fusarium on dead seeds	0	0	6	4	4
severe symptoms	0	4	46	35	2
light symptoms	2	60	27	33	7
total *Fusarium*	2	64	73	68	13
epiblast browning	4	23	7	11	19
abnormal seedlings	7	1	0	1	4
non-germinated (dead) seeds	5	4	0	0	1

ed as a still lighter symptom of *Fusarium* was thereby disproved. If the rootlets with symptoms of all samples would have been plated, yet another fact about *Septoria* would have emerged. Now this did not become known before completion of these series. It was then noted that certain samples gave rather high root rot percentages in blotter tests, but that the *Fusarium* percentages found on agar were much lower. Invariably such samples had high *S. nodorum* levels.

In the 1967 harvest samples very low *Fusarium* percentages were sometimes found, even on peptone-PCNB agar without NaClO pretreatment. The same samples gave rather high root rot percentages in the blotter test for Fusarium (table 110). When the brown rootlets from these blotter tests were plated on

TABLE 110. Infection percentages found in 6 wheat seed samples with different seed health testing methods

sample	*Fusarium* spp.			*Septoria nodorum*
	PDA after NaClO treatment	peptone-PCNB agar without NaClO treatment	routine blotter method	PDA without NaClO pretreatment
1	4	6	11	22
2	3	3	16	25
3	0	6	21	32
4	9	8	21	16
5	3	3	30	21
6	3	11	39	30
average	3½	6	23	24½

PDA, the larger part gave rise to colonies of *S. nodorum*. A further study showed that *Septoria* behaves very erratic in this test, causing light symptoms in some samples and none in others. This was found not to be related to wheat variety.

In the second series of samples *S. nodorum* appeared to be much less involved in the epiblast symptoms, showing no correlation at all. Apparently these symptoms now were almost exclusively due to *F. nivale*.

The conclusion can be drawn that in this blotter test a single symptom may have different causes and a single pathogen may cause different symptoms.

The effect of NaClO pretreatment on the correlations is interesting, though differences are small. NaClO pretreatment tends to decrease the correlation between blotter test symptoms (notably the light ones) and *F. graminearum* and to increase it for *F. nivale*. NaClO pretreatment in the blotter tests severely reduced the percentage of *Fusarium* infections found, the remaining significant correlations being mainly those with *F. nivale*. The correlation between epiblast browning and *S. nodorum* disappeared to make place for a similar one with *F. nivale*. Surface disinfection reduces the inoculum potential (see section 6.3). Epiblast browning caused by *S. nodorum* apparently requires a high inoculum potential whereas *F. nivale* causes epiblast browning already at low inoculum levels, causing root rot symptoms at higher ones.

In section 5.2, mention was made of the effect of temperature and soil antagonism on the significance of blotter test symptoms (blotter disc experiment). In agreement with that was the observation that when seeds were sown in silty clay loam in the greenhouse kept at 15°C during the wintermonths, *F. nivale* did not cause much trouble. The coleoptyle lesions produced could for the larger part be attributed to *S. nodorum* although sometimes *F. graminearum* could also be isolated.

Whereas lesions produced at 15°C have a high correlation with *S. nodorum* (table 107), *F. graminearum* is correlated with emergence (tables 106 and 107). This may be partly accidental as it is also highly correlated with the group of dead seeds carrying *Fusarium*. In the past we called this group 'killed seeds' but since we found that NaClO pretreatment in the blotter test reduces its percentage, this indication was changed. Part of this group of dead seeds with *Fusarium* therefore may consist of dead seeds afterwards contaminated by *Fusarium* spp., and another part of really killed seeds.

If the temperature is lowered, the above soil-disease symptoms can be produced by other fungi than *Septoria*, e.g. by *F. nivale*. The difficulty is to distinguish between the two. It was found (by agar plating) that lesions consisting of very

fine stripelets could always be attributed to *Fusarium*, the solid ones mainly to *S. nodorum*. Though this latter group still included much *Fusarium* this distinction was the best one possible.

An experiment was run at 7 °C in steamed greenhouse soil in a cooled greenhouse section of the Agricultural University. The results presented in tables 105 to 107 indicate the role of *F. nivale* on emergence and disease development. The low correlation with stripes can be explained by the fact that many were lost in the *Septoria* symptoms.

The percentage emergence in the jiffy pot experiment (11-B) again gave a very good correlation with *F. nivale*. *F. graminearum* was giving a lower correlation with emergence in the case of the 7 °C experiment, perhaps because of the increased influence of *F. nivale*.

The effect of temperature is also illustrated by two experiments given in the tables 111 and 112. These figures show that *F. nivale* caused few above ground lesions but may appreciably reduce emergence at low temperature. *S. nodorum* caused a lot of visible lesions which at lower temperature were more associated with seedling abnormality and scarcely reduced emergence.

If in the above tests emergence in soil was compared with the group of dead seeds in the blotter test good correlations were always found, but those with the other blotter test symptoms depended on circumstances. The normal blotter test without NaClO pretreatment gave the better correlations with soil performance. The agar test is more informative, as the outcome of various weather conditions can be better predicted from a knowledge of the percentages of the various *Fusarium* species and of *S. nodorum*.

At Wageningen the official germination tests for wheat seed are carried out

TABLE 111. Effect of temperature on the average percentages of disease symptoms obtained in soil tests (silty clay loam) with 5 wheat seed samples containing mainly *F. nivale* (averages on PDA after NaClO treatment: 32% *F. nivale*, 1% *F. graminearum* and 3% *S. nodorum*)

	15°C	12°C	7°C
healthy seedlings	62	58	52
Fusarium lesions	9	12	7
Septoria lesions	9	10	8
abnormal with *Septoria*	2	2	5
abnormal (not diseased)	0	1	3
total emergence	82	83	75

TABLE 112. Effect of temperature on the average percentages of disease symptoms obtained in soil tests (silty clay loam) with 5 wheat seed samples containing mainly *Septoria nodorum* (averages on PDA after NaClO treatment: 4% *F. nivale*; ½% *F. graminearum* and 36% *S. nodorum*

	15°C	12°C	7°C
healthy seedlings	45	52	46
Fusarium lesions	3	3	4
Septoria lesions	40	32	24
abnormal with *Septoria*	1	3	12
abnormal (not diseased)	2	1	3
total emergence	91	91	89

in silversand at 20°C after a few days at 10°C for after-ripening. Diseased seedlings of which the coleoptyles are rotted through are considered as abnormal ones and not included in the germination percentage. For the 1965 samples the percentage germination was also determined in this way and the results are given in table 102. It was found that the symptoms usually attributed to *Fusarium* spp. were for the larger part caused by *Septoria nodorum* and for a smaller part by *F. graminearum*, *F. culmorum* and *F. avenaceum*. *F. nivale* escapes observation altogether in the silversand test for germination.

The correlation of the germination figures with those of soil emergence was better at higher soil test temperatures than at lower ones (table 106). This was due to the presence of much *F. nivale* in the samples. It can therefore be concluded that if this fungus is present in the seed, germination figures give no good indication of the seed value if it meets low temperature conditions in the field.

The above observations are in agreement with those of GERM (1960), KIETREIBER (1966) and FRITZ (1967).

The routine blotter method for *S. nodorum* (Kietreiber method) is a good one for those cultivars that produce protuberances. This seems to be the case for most Austrian winter wheat cultivars but not for many others (KIETREIBER, 1966). This means that one has to rely on other symptoms such as abnormalities of the seedlings or coleoptyle lesions. This makes things very complicated because *Fusarium* may also be involved.

At the inspection of the *Septoria* blotter test a large number of symptoms was noted, which were later combined to give the data of table 99.

Plating experiments carried out on a small scale showed that the presence of protuberances (group a) always indicated presence of *S. nodorum*, but *Fusarium*

species were often present at the same time. *Fusarium* spp. were most frequently isolated from the seedlings with coleoptyle stripelets and those with stunted roots (group d and e), but *S. nodorum* was sometimes obtained as well. From coleoptyle lesions (group b) both *Fusarium* and *Septoria* were frequently isolated. It was hard to express these plating results in exact figures due to frequent occurrence of *Mucor* spp. It became clear, however, that it was nearly impossible to distinguish *Septoria* and *Fusarium* symptoms from each other. The averages for group a are not always giving a good picture as wheat varieties differ in their ability to produce protuberances. In cultivars in which the latter are easily obtained (e.g. Tadorna), higher *S. nodorum* percentages are often found in the blotter test than on agar. The agar test gives too low percentages for this infection anyway (cf. sections 5.7 and 6.3).

The proportion of *S. nodorum* included in group b is hard to judge. One and other will vary from sample to sample. This is also shown by the large difference between the two series for i, constituting a guess for the *Septoria* percentage. The second series is apparently much more influenced by *Fusarium*. The sum given at j constitutes a guess for the *Fusarium* percentage and happens to correspond rather well with that of group d of table 98. As we have seen the latter figure itself may be affected by *Septoria* too.

Referring back to the blotter method for *Fusarium* it can be said that a low temperature test at continuously 10°C produces only severe symptoms. Interference by *Septoria* which also produces seedling abnormality will moreover increase.

When comparing the various *S. nodorum* data for blotter, agar and greenhouse test (coleoptyle lesions), we are confronted with the difficulty that all these figures are relative and moreover, with the exception of the agar test, highly uncertain. In the agar test the highest figure must be the best. For wheat varieties that always produce protuberances the same can be said for the blotter test which in that case is probably the best one. The greenhouse test has the disadvantage that the lesions are not always necessarily due to *Septoria* whereas soil factors may also affect them.

7.4 DISCUSSIONS

Due to the additional influence of edaphic factors the set of environmental conditions affecting the seed + pathogen combination in the field is even much more complex than that encountered in laboratory incubation tests. Together with the fact that most of the environmental factors encountered in the field

are highly unpredictable (especially in climates like that of Western Europe), this explains why the effect of the presence of a pathogen on its host after sowing is hard to tell.

As field conditions cannot be controlled it is logical that one should try to substitute them by conditioned soil tests. In such tests the most favourable conditions for *disease* development – in contrast to those for *pathogen* expression in laboratory tests – can be given. The problem remains, however, that soil as used in these tests is never like natural soil in the field.

The above explains the difficulties involved if one wants to establish correlations between results of incubation methods for seed health and disease development in the field. For germination capacity of seeds matters are more simple, as in general we have here only to do with the reaction of the host plant to the environmental conditions provided. In as far the correlation coefficients do not approach 1.00 this is due to the fact that conditions become less optimal than provided in the germination test and to the presence of seed or soil-borne pathogens.

When trying to obtain correlation figures in relation with seed health we have to compare the outcome of 'more or less' standardized incubation conditions on the seed biotope and the outcome of the effect between the latter and unpredictable environmental factors in the field. This may explain why but few such correlations have been determined and why they are usually low.

There are exceptions such as those for smut infections of barley and wheat (covered smut of wheat: HEALD, 1921; loose smut of barley: RUSSELL, 1950; RUSSEL & POPP, 1951; MORTON, 1960; loose smut of wheat: POPP, 1959). It should be noted that these North American data were obtained in areas where soil and weather conditions are rather uniform. MORTON (1960), for instance, found a correlation of 0.95 between *Ustilago nuda* infection of barley seed as determined by the embryo method and loose smut development in the field. It should be observed that here the young plant actually is already infected (embryo). Another point is that these infections have the advantage that disease development can be easily measured and attributed exclusively to seed transmission. Yet even here the proportion of loose smut can be influenced by ecological factors (DOLING, 1965).

As pointed out in the beginning of the previous section the infections used for correlation experiments discussed in it were a complex entity by themselves. The reasons for using them were given in section 7.1, the main one being the dissatisfaction with existing methods for those infections. We have seen in section 7.3 that much was learned by these experiments about the background

of the methods used.

The complexity is due to the fact that e.g. emergence is affected by many other factors than e.g. the percentage of dead seeds with *Fusarium*, as found in the blotter test. It will be clear that the latter is only one of the emergence restricting factors. Consequently, for the first series of samples a combination of the *Fusarium* infected dead seeds with *Fusarium* free abnormal and dead seeds and with the group of severe symptoms together gave the relatively high correlation of −0.71 with emergence in the greenhouse (cf. table 105).

The seed trade would like to use quantitative data clearly indicating the immediate consequences for the crop, somewhat like germination data. Seed health data are, however, only indicating a risk for the case that the seed or seedling meets conditions favourable for the pathogen(s) carried by it.

We know for example, that seed-borne *Fusarium* may reduce emergence (FIALA, 1964; COLHOUN & PARK, 1964; DE TEMPE, 1958). But it makes no sense to try to establish potential losses from seed health data on this basis, as these may vary with time and place.

TABLE 113. Correlation coefficients between the official *Fusarium* blotter test results and field emergence (spring) for a number of wheat seed samples of 1955, 1956 and 1957. Data supplied by DE TEMPE

blotter test category	field emergence for samples of		
	1955	1956	1957
Fusarium on dead seeds	−0.21	−0.30	−0.35
severe symptoms	−0.51	−0.35	−0.58
light symptoms	−0.62	−0.39	−0.36
total *Fusarium*	−0.71	−0.41	−0.46
significant on 5% level	0.35	0.25	0.38
1% level	0.49	0.35	0.54
number of samples	35	72	28

From old data of DE TEMPE (1958) on *Fusarium* infected wheat seed samples the correlation coefficients of table 113 were computed. To these can be added those of table 106, so that correlation data over 4 years can be compared. It can then be noted that the correlations vary from year to year. The same even appears from the correlation data of WIESNER (1965) for the relation between emergence in virtually sterile silversand and presence of *Phoma betae* in sugar beet seed.

The effect on seedling emergence is often more easily measured as disease symptoms are often hard to observe or identify. Yet the effect on seedling emergence may not be the most important. In so far as the pathogen effect is not already included in figures for germination capacity (e.g. group of dead seeds with *Fusarium*), it may be regarded as a correction on germination figures. The effect on emergence is moreover, often compensated by increased development of the remaining plants (MACHACEK *et al.*, 1954), although this does not apply for such a case as *Botrytis cinerea* in fibre flax seed, for which DE TEMPE (1968b, 1961b) found a good relationship between laboratory and field results. In the latter case the pathogen will under favourable conditions spread from an infected plant and kill many surrounding plants (VAN DER SPEK, 1965).

The effect of seed transmission on later disease development in the field may be much more important. In this connection light infections can sometimes be more important than severe ones. Severe infections may merely cause pre-emergence death whereas light infection may give rise to diseased plants serving as potential foci of inoculum in the field. This may be the case with *Septoria nodorum*, of which very light infections (eliminated by NaClO pretreatment in agar tests) were found to be capable of causing coleoptyle lesions in soil. The further importance of such lesions is hard to measure quantitatively as other sources of inoculum may be involved.

BRÖNNIMANN (1968) could not find any definite relationship between wheat seed infection by *Septoria nodorum* on one hand and the 1000 grain weight, the number of grains per ear, or the infection of the harvested seed on the other hand. He did note, however, in some cases an effect of seed disinfection and states that the seed infection is in the first instance important for seed transmission from year to year. It makes no difference whether the seed lot is only lightly or heavily infected, as a very light infection does already suffice.

Apart from their importance as foci of inoculum light infections may also result in qualitative losses of the harvested crop. An example is the presence of spotted peas due to *Ascochyta* spp., which reduces the market value of the seed lot.

Correlation coefficients will moreover be hard to establish for diseases such as those caused by seed-borne *Phoma* in corn-salad, beet and cabbage. In section 4.6 we saw that the pathogen may stay with the plant throughout its life, attacking it whenever environmental conditions are reducing the host vigour. LEBEN (1965) speaks of the resident phase and supplies a number of similar examples of plant pathogens. During this phase the pathogen may build up its inoculum energy or number of infective units. This means that under favou-

rable conditions even slight infection can become important later in the growing season, when their seed-borne origin may be difficult to prove.

DEKKER (1957) observed symptomless pea plants carrying *Ascochyta pisi* and producing infected seeds. TIFFANY (1951) found the same for *Colletotrichum truncatum* in soybean. Such symptomless infection might be an efficient way of ensuring a continuation of the cycle plant-seed-plant and may be seen as an aspect of a highly evolved parasitism.

BAKER & SMITH (1966) give a number of reasons for the significance of seed infection. These amount to the fact that seed transmission is one of the most efficient ways of dispersal for a pathogen, because of the intimate association of pathogen and host, both in space and time. Not only can a pathogen, new to an area or field, be introduced with the seed, but also if it is already present, seed-borne inoculum may cause a more rapid building-up of inoculum. It is moreover distributed at random, over the field, possibly giving rise to many foci of primary infection. BAKER & SMITH (ibid.) also remark that a pathogen strain with a marked virulence for the given host variety is preferentially selected in the seed field. The importance of the occurrence of seed-borne pathogens is often minimized by the idea that the pathogen is widely distributed anyway, this selective process not being realized. Examples given by the above authors from the literature are the selection of virulent strains of *Rhizoctonia solani* for pepper and tomato, and of *Fusarium solani* f. *cucurbitae* strain for squash fruits.

Such factors are hard to express quantitatively. The right attitude therefore seems to be to refuse to run these risks and to demand healthy seed. The presence or absence of the pathogen is therefore in many cases more important than exact infection figures.

Correlation with soil tests would remain important, however, for comparing one method with another and to decide about the method that best indicates field performance.

8 DISCUSSION

8.1 FACTORS AFFECTING THE SEED MICROFLORA

Though the present work was started with the purpose to investigate the cause of the variations in results occurring in incubation tests, the first part of this discussion will in a general way treat the seed as an ecosystem, or biotope. This

ecosystem consists of two components, viz. the host seed and its microbiota. The microfauna and viruses if present will not be discussed, and only the microflora is considered here.

The seed-borne pathogens which form a part of the seed microflora will be especially taken into consideration. They are to a high extent influenced by the environment and have therefore to be considered in relation to this environment.

It is well known that seeds carry a rather characteristic microflora which varies with the host species. This is among others apparent from numerous reports on organisms isolated from various seeds. PONCHET (1966) remarks in connection with his studies on the etiology of the mycopericarpic communities of the wheat caryopsis, that the latter and especially its pericarp are nutritive substrates that are suitable only for certain species. In view of the large number of air-borne fungal species that might enter the wheat caryopsis the establishment of only a few of them indicates a highly selective process being operative during development and maturation of the seed.

Differences between the microfloras of seed lots can be thought to be related to host species or cultivar. This is especially true for the deeper seated microfloras as on the surface many accidental guests may be carried as well. MALONE & MUSKETT (1964) mention e.g. the frequent occurrence of chlamydospores of *Ustilago anomala* on oat seeds. This fungus causes smut on *Polygonum* spp. but is innocuous for oats.

The normal microflora may partly arrive on the developing seed by air currents or via splashing rain from other hosts or from the soil. A part of the seed flora can be considered as typically epiphytic. This is especially true for many bacteria and yeasts (LEBEN, 1965). Some fungi such as *Ustilago, Helminthosporium, Alternaria* and *Epicoccum* spp. are associated with the host plant throughout its growing period (MALONE & MUSKETT, 1964). Parasitic or saprophytic microorganisms may get into the flower and be borne at the surface of the seeds as external contaminants or they may penetrate into its external layers, inducing 'transitional infections' (CICCARONE, 1958; GAÜMANN, 1951). More accidental seed coat inhabitants may be picked up during harvesting and storage from soil, dust, machinery, storage bins, etc.

It can be assumed that growing conditions in the field, such as cultural methods (use of fertilizers, fungicides, herbicides, etc.), soil-type and fertility, area and season of growth are also important. These factors will affect the host vigour, duration of certain growth stages (flowering, seed set and maturation), lodging, etc., as well as the quantity and kind of available inoculum.

Most important after host species seem to be the climatic factors. High humidity and rainfall favour the establishment of a large number of pathogens in seeds (cf. KREITLOW *et al.*, 1961). It has been the reason for the shift in seed production areas in North America from Michigan, New York and the New England states to dry areas such as Oregon, California and Idaho. Wet weather during harvest time may also be important. An example thereof is among others given by MEAD & CORMACK (1961) who found this to be the case for *Ascochyta imperfecta* in alfalfa seeds harvested in Manitoba.

It is also a common experience at the Wageningen and other seed testing stations that after wet growing seasons increased percentages for many infections can be expected. This is among others the case for *Botrytis cinerea* infection of flax seed. Interestingly enough, however, its incidence after the really extremely wet seasons of 1965 and 1966 was quite low. It would be interesting to know whether this would be somehow related to bacterial antagonism (cf. ch. 3). The cereal seeds of those seasons contained high percentages of *Fusarium* spp. but that harvested after the relatively dry season of 1967 only low percentages. The incidence of *F. poae* which is usually quite low in Dutch samples was, however, very much increased (cf. section 6.6).
HEWETT (1965, 1967) also related rainfall with seed infection percentages found for *Fusarium* and *Septoria* in wheat seeds.

PONCHET (1966) found that in case of the wheat caryopsis climatic factors, notably humidity, determine the possibility for some fungi to enter. He made a distinction between hydrophytic parasites *(Fusarium* and *Septoria)* which are favoured by wet conditions, and mesophytic saprophytes *(Alternaria, Epicoccum* and *Cladosporium)* which are more indifferent for humitity. Competition between these fungi was found to be another factor, those that enter first having a competitive advantage and usually becoming the dominant species present.

During storage of the seeds some organisms may decrease or die out whereas others may be stimulated. The latter is especially the case with the so-called storage fungi (notably xerophytic species of *Aspergillus* and *Penicillium*) causing deterioration of stored grain. The effect of various factors on their growth and development has been especially studied by the school of CHRISTENSEN at Minnesota (e.g. CHRISTENSEN, 1957). Temperature and humidity were the main factors, certain species becoming dominant under specified conditions.

WALLEN (1964) has given a review of the literature on the longevity of seed-borne pathogens. He points out that unfortunately most of the literature does not pertain to optimum conditions fo seed storage or rigidly controlled conditions, so that sometimes contradictory results are not surprising. It might be

added that the form of the pathogen, its depth of penetration and inoculum potential may also be important.

One of the conclusions that may be drawn is that the composition of the seed flora changes with the age of the seed. This applies to saprophytes as well as parasites. Differential survival rates of pathogen and saprophyte were suggested in section 5.9 as an explanation for the increased percentages sometimes found in seed health tests for *Helminthosporium avenae* in oat and *Septoria nodorum* in wheat seed.

Differential survival of host and pathogen is sometimes used for control purposes by sowing seeds older than the life span of the pathogen. An example is the control of *Septoria apiicola* in celery seeds by the use of seeds being more than two years old.

It may be interesting to note here that CROSIER (1963) used seeds for the storage of various fungi. He inoculated 72 fungus species from macerated agar cultures into the florets of barley, oats, rye and wheat. In this way he obtained barley and wheat seeds infected with e.g. *Ascochyta pinodella* (from peas), *Dendryphion penicillatum* from poppy, and *Diaporthe phaseolorum* from lima beans.

The species belonging to the seed microflora can be divided into three broad groups, based on their relationship to their host, viz., the pathogens (A), symbionts (B) and saprophytes (C). These groups will now be discussed in the same order.

A. – Pathogens

The effect of the pathogen on the host seeds has been reviewed by WALLEN (1964). For our purpose we recognize four categories, viz.:

1. No normal seed is produced, but the seeds have been largely replaced by fungal tissues (ergot, bunt and smuts) or they are of small size and shrivelled (*Septoria nodorum* in wheat). In both cases their formation reduces the seed yield, but they can be largely removed by seed cleaning.
2. The seed is killed before germination but is sufficiently normal in size and shape as not to be removed during seed cleaning (*Gloeotinia temulenta* in ryegrass seed). The presence of such pathogens is always expressed by reduced germination capacity.
3. The pathogen causes pre-emergence death. This may or may not be incalculated in germination figures as this depends on conditions present during germination. In contrast to the former two categories seed treatment may here increase germination and emergence.
4. The seed gives rise to diseased seedlings or plants which can serve as centres

of inoculum in the field. This category may be considered as the most important one. It is this group in which seed transmission becomes an efficient way of transport through time and space and on which the considerations for the importance of seed transmission as enumerated by BAKER & SMITH (1966) and mentioned in section 7.4 apply.
It should be realized of course that one and the same pathogen may produce more than one or even all of the above effects, such as e.g. *Fusarium* and *Septoria* infections of wheat seed.

B.–Symbionts

Besides pathogens seeds may also carry symbiotic organisms. In contrast to seed-borne diseases one can then speak of seed-borne symbiosis (LANGE, 1966), sometimes less appropriately called hereditary symbiosis (VON FABER, 1912, 1914). Unfortunately very little is known as yet about this group of seed inhabitants. Examples of seed-borne symbiotic bacteria are *Bacteria foliicola* in seeds of *Ardisia* spp. (N-fixation, MIEHE, 1914 to 1919; DE JONGH, 1938), bacteria in seeds of various Rubiaceae (VON FABER, 1912, 1914), seed transmission of *Rhizobium* in alfalfa (ASH & ALLEN, 1948). A fungal example may be that of *Phomopsis casuarinae* in *Casurina equisitifolia* (BOSE, 1947). Various reports on endophytic fungi in *Lolium* and *Festuca* seeds are less certain as to their symbiotic significance (MCLENNAN, 1920; GRÜNNEWIG, 1933; NEILL, 1941). They have been suggested to assist the nutrition of the developing embryo. Various endophytic organisms are known to occur in many plant species and they are generally supposed to have a beneficial effect on plant growth. GELTSER (1965) claims that endotrophic mycorrhiza occur also in the seeds and that they do not enter the plant from the soil, but that they are seed-transmitted in nature. That these mycorrhiza do not develop in sterile soil was shown to be due to the lack of certain growth stimulants normally produced by the soil microflora.

Alternaria tenuis has here been considered to belong to the next group, that of the saprophytes. BOSE (1956) considers it, however, as probably useful for its host, although it may sometimes behave as a weak pathogen. But there is no sharp demarcation between symbiosis and parasitism, both phenomena having much in common, morphologically as well as physiologically. PONCHET (1966) also claims a stimulatory effect of seed-borne *Alternaria* spp. on wheat seedlings, possibly due to the production of gibberellin-like substances.

C.–Saprophytes

Under the saprophytic microflora of the seeds we understand here organisms

that are neither pathogenic nor symbiotic. It includes a large group in which we can distinguish typical field fungi such as *Alternaria, Cladosporium*, etc., from typical storage fungi such as certain species of *Penicillium* and *Aspergillus*. The latter are definitely injurious. Part of the saprophytic flora is practically always found to be present on certain seeds. An example is the occurrence of *Alternaria tenuis*, *Epicoccum nigrum* and *Cladosporium herbarum* in wheat seeds. That the bacterial flora is also rather specific was already shown by DÜGGELI in 1904. WALLACE & LOCHHEAD (1961) described various morphological groups from seeds of wheat, oats, clover, thyme, alfalfa and flax.

The saprophytic seed inhabitants may have various effects on their hosts, depending on circumstances. They may e.g. reduce seed viability and vigour under poor weather conditions at harvest time (field fungi) or during storage at a too high temperature and humidity (storage fungi). Various saprophytic fungal seed inhabitants have been reported to invade tissues of germinating seeds and to kill seedlings (HARRIS & ELLETT, 1945; GIBSON, 1957).

VERONA (1963) discusses the importance of the seed-flora for the development and composition of the rhizosphere flora, giving pertinent references to the literature. He uses the term spermosphere in this connection, which is a modification of the term spermatosphere introduced by SLYKHUIS (1947). VERONA defines it as 'a microhabitat which surround the germinating seed'. It is determined by two parameters, viz. the seed and its microflora on the one hand and the medium in which the seed germinates (normally the soil) on the other hand.

A very important aspect of the saprophytic microflora may be furthermore its protective action against seed- and soil-borne diseases. PONCHET (1966) investigated the factors affecting the establishment of the microflora of wheat seed and found competition for space and hegemony to be important, invasion by saprophytic fungi limiting invasion by parasites. At germination similar processes play a role, resulting in more or less disease development. According to PONCHET (l.c.) at temperatures below 12°C the biological balance shifts in favour of the parasites *Fusarium nivale* and *Septoria nodorum*, at higher temperatures the growth of the plantlets and the development of the saprophytes *Alternaria* and *Epicoccum* contributing to their natural protection is favoured. TVEIT & MOORE (1954) claimed that the resistance of some Brasilian oat varieties to *Helminthosporium victoriae* is related to the occurrence of two *Chaetomium* spp. in their seed flora. LEDINGHAM *et al.* (1949) also suggested the possibility that the differences between the susceptibility of host varieties to certain pathogens might be related to differences in composition of the seed flora. Interesting in this connection is the observation by RANGASWAMI & RAMALIN-

GAM (1962) that *Helminthosporium oryzae*, when added as a conidial suspension to sterile and non-sterile soils, survived for 131 and 98 days respectively. When it was added on rice seed it survived for 37 days in sterile soil and for 15 days in non-sterile soil. Our own observations on the difference in effectivity of wheat seed inoculation with and without NaClO pretreatment are in agreement with this, as well as similar reports by LEDINGHAM *et al.* (1949) for *Helminthosporium sativum.*

Extra inoculation with natural seed inhabitants (*Alternaria* and *Epiccocum*) was also shown to decrease disease incidence by *Helminthosporium sativum* in barley seedlings (CSUTI *et al.*, 1967) and by *Fusarium nivale* in wheat (section 5.8). Artificial inoculation of seeds has in fact been used to control certain diseases, examples of which are the bacterization of seeds (NOVOGRUDSKII, 1937; BERESOVA & NAUOMOVA, 1939; KRASILNIKOV & RAZNITZYNA, 1946). and inoculation with saprophytic fungi (TVEIT & WOOD, 1955).

Up till now we have been mainly considering only one of the components of the seed biotope, viz., the seed microflora. It is generally accepted that environmental conditions during seed development, maturation, harvesting and storage effect its viability and vigour. It will be clear from the preceding part of this discussion that this is for a large part via the effect of those conditions on the microflora. It is also generally known that reduced host vigour makes plants more susceptible to most parasites. Exceptions are the parabiotic parasites (GÄUMANN, 1951). GÄUMANN (l.c.) also points out that the first and the two last steps in the chain of events involved are usually known to us, viz., the vigour-reducing agent, the reduced vigour itself and the increased disease development. In section 4.13 we pointed out that one of the factors involved in decreased vigour might be increased leaching of nutritive substances from weakened seeds. HOTTES & HUELSON (1927) found that maize seed of low vigour and giving poor soil emergence yielded large amounts of exudates when soaked in water, which he measured optically. TATUM (1954) found the same relation for maize seed and PRESLEY (1958) for cotton seed. The latter used electric conductivity of the soak water as a measurement for vigour. He found that prolonged soaking decreased seed vigour and promoted later fungal growth (cf. positive soak effect, section 3.16). MATTHEWS & WHITBREAD (1968) used electric conductivity of soak water as an indication for vigour of pea seed, seeds producing much exudates usually failing in soil. TAKAYANAGI & MURAKAMI (1968) used a colorimetric method for sugars in exudates of steeped cabbage seed as an indication of viability, dead seeds producing the largest quantity.

Seeds do ordinarily secrete various substances when moistened and during germination. This is probably true for all plant parts and it provides the basis for the existence of epiphytic microorganisms. VERONA (1963) mentions the influence of such exudates and divides them into two categories, viz. inhibitory (toxic) and stimulatory ones. In the latter category he distinguishes nutritive substances and growth factors. He also claims an agglutinating effect of mucilaginous substances present around many seeds (e.g. *Linum*, *Plantago*, *Lepidium*) on microorganisms, although these substances may be more or less rapidly decomposed by microbial attack.

Knowledge of seed diffusates toxic to microorganisms by their content of antibiotic substances (phytoncides) is summarized by BOWEN (1961) after a short review of pertinent literature as follows: 'Antibiotic seed diffusates occur with a wide range of plants and these are active against plant pathogenic fungi and gram-positive bacteria.' Such toxic substances may very well be another important factor affecting the composition of seed floras and may explain the rather rare occurrence of gram-positive bacteria on seeds. They may also have consequences for the survival of organisms inoculated to seed, such as e.g. *Rhizobium* spp. (BOWEN, l.c.). Such substances may also be related to resistance against certain diseases.

Exudates that stimulate fungal growth may also be involved in disease resistance by affecting the composition of the spermosphere. SCHROTH & COOK (1964) showed for instance that the resistance of varieties of bean to damping off caused by *Rhizoctonia solani* and *Pythium* spp. bears some relation to the amount of substances diffusing from soaked seeds that give positive reactions with ninhydrin and silver nitrate. The same applies to the importance of sucrose released from pea seeds in relation to damping-off by *Pythium* spp. (FLENTJE, 1959).

One thing will have become quite clear, namely the extreme complexity of the factors affecting the seed biotope during its establishment, storage and germination. This corresponds with the findings on factors affecting seed health tests as reported in the preceding chapters. Environmental factors, for instance, play a very important role. They influence, however, in first instance the biotope as a whole. Their effect on individual members, e.g. a certain pathogen is often much more indirect. The low-temperature effect on pathogenesis by *Fusarium nivale* (section 5.2), for instance, is not due to low temperature being optimal for its growth, but because it is less favourable for many competitors. The complexity of the interactions can be seen from the fact that the seed is somewhat selective in choosing its own microflora. At the same time this microflora may

affect seed vigour. At lower vigour levels weaker parasites may get a better chance too. Hence pathogens may face increasing competition, etc. In the foregoing chapters we have tried to separate such factors as bacterial antagonism, host vigour, interfungal antagonism and inoculum potential, but it should be evident that this could only be done by often oversimplifying the matter.

In increased bacterial antagonism due to wet conditions for instance, seed exudates, host vigour and inoculum potential must have played a role. Soaking of seeds in water stimulates fungal growth, but when bacteria develop faster, the fungi are at a disadvantage. Seed vigour on the other hand is decreased.

The elimination or reduction of one factor increases the importance of another one. Reduction or elimination of host vigour increases bacterial as well as fungal antagonism (soak-effect, freezing); elimination of bacterial antagonism increases interfungal antagonism. Elimination of part of the fungal flora (e.g. dry heat method, section 5.5) may increase interference between the remaining ones. In the highly selective peptone-PCNB agar method competition between different *Fusarium* spp. present on the same seed was observed.

An important factor involved is that of inoculum potential (ch. 6). An organism that succeeds in getting the lead over another one, is at an advantage. This situation is favoured by a high inoculum potential, a higher one being useful for a more rapid start and earlier colonization of available substrates.

A deeper penetration is also important and can be considered to be associated with the presence of a higher inoculum potential. Organisms that have succeeded in penetrating the seed more deeply will be less hampered by competition but may still have to cope with host resistance (e.g. *Ascochyta* spp. in peas). Only certain pathogens penetrate deeply into the seeds unless host vigour decreases.

It will be evident that the outcome of incubation methods for seed health testing is affected by a complex set of factors. The practical consequences thereof will be discussed in the next section (8.2).

8.2 Practical consequences for the choise of seed health testing methods

During the study of the biotic factors involved in seed health testing methods, various techniques were tried or used to reduce, eliminate or avoid such factors (antibiotics, freezing, selective compounds). The effects were surprising in that they often resulted in considerably increased infection percentages. This proved the importance of the eliminated factors. It also indicated the reasons for dif-

ferences in level between the results of various methods, e.g. agar and blotter test for *Botrytis cinerea* in flax (ch. 3), *Phoma betae* in beet (ch. 4) and *Helminthosporium avenae* in oat seed (ch. 5).

From a biological standpoint this is very interesting, but for the practice of seed health testing it calls forward the question of the relative importance of the results obtained. In order to simplify dicussion on this subject we propose to divide the incubation methods into two groups, viz. in saprophytic and pathogenic methods. In the first group host vigour does not play a significant role, in the second one it usually does. Pathogenic methods require the pathogen to attack, or develop in the young seedling and to cause disease symptoms. Examples are all sand and soil tests as well as certain blotter tests. In saprophytic methods the growth of the pathogen is observed directly. Examples are the agar and freezing methods. In the freezing method the pathogen may, however, be given a certain lead over saprophytic competitors by regulating pre-freezing incubation conditions.

If we call a method more pathogenic than another one, we mean to say that the pathogenic capacities of the pathogen play a proportionally larger role. The normal blotter test can, for instance, be considered to be a more pathogenic one than a 2,4-D blotter test in which the pathogen can more easily develop and fructify on the host tissues so that the relative importance of disease symptoms decreases, whereas that of signs of the pathogen itself increases.

In the normal blotter test for *Fusarium* spp. in wheat seed, for example, the light and severe root rot symptoms are the main ones indicating infection. In the 2,4-D blotter test the presence of *Fusarium* mycelium and fructifications is looked for. Similarly the normal blotter test for *Fusarium* in cereal seeds can be considered a more pathogenic one than that for *Helminthosporium* spp. in the same seeds.

In the latter test signs of the pathogen itself are looked for, and disease symptoms scarcely occur as the pathogen grows mainly on the dead outer seed structures.

The preceeding discussion on pathogenic and saprophytic methods has been given because it might be argued that pathogenic methods give the best information about sowing value. The reason for this is their greater similarity with the field. It could also be pointed out that they automatically incalculate such factors as seed-borne antagonism and host vigour. These arguments can be answered by saying that a pathogenic method can always be considered as more or less pathogenic.

The absolute standard would be field sowing. But in the field we cannot con-

trol environmental conditions so that we have to be satisfied with conditioned soil tests. These have in fact sometimes been advocated for general use (MEAD *et al.*, 1950; MACHACEK & WALLACE, 1942). Such tests are, however, laborious, time-consuming and hard to standardize. The use of non-sterile soil introduces another complex of variables, every soil being different and changing with conditions. If we use tests in sterilized soil or in virtually sterile silversand, we are getting further removed from natural conditions, quite apart from possible side effects of soil sterilization. We are, moreover, still faced with many of the same problems, one of them being the fact that disease symptoms may often be impossible or difficult to obtain and to identify (errors of judgment). The next step is the use of blotter tests of which we do not need again to discuss the difficulties that may be involved in their standardization and which led to the idea of removing biotic factors as much as possible.

If one pleads, therefore, for tests representing field conditions as much as possible it will be evident that a rather arbitrary choice must be made. It will also be clear that it generally involves sacrificing such factors as simplicity, economy and especially reproducibility.

The alternative is the use of methods in which interfering biotic factors are as much as possible reduced, eliminated or circumvented. In its most extreme form this approach will lead to highly selective methods in which the entire seed biotope is eliminated, except for the pathogen. A rather good example is the peptone-PCNB method for *Fusarium* spp.

It will be clear that this simplifies the standardization of incubation conditions, as those most suitable for growth and development of the pathogen can be selected. Inspection will also be simplified as other organisms are absent. It follows that this approach offers the best prospects for simple, cheap and reproducible methods needed for official routine work and international trade. Such highly selective methods are not as yet available for most infections. It is to be expected, however, that selective chemicals and methods will become increasingly available if the present trend continues.

In the meantime we have to use techniques that are less selective but which may often be very good, greater selectivity being even unnecessary. Examples are the use of antibiotics in the blotter test for *Botrytis cinerea* in flax seed (ch. 3), agar tests with NaClO pretreatment for *Phoma betae* in beet and *Ascochyta* spp. in pea seed (ch. 4), the freezing method for *Helminthosporium* spp. in cereal seeds (ch. 4), the use of oxgall-PDA for *Septoria nodorum* in wheat seed (ch. 5), etc.

The objection against the elimination of seed-borne antagonists by various

selective methods can be answered by the argument that antagonism as encountered in seed health tests will be affected by incubation conditions, and so impairs the reproducibility of the test. Furthermore the incubation conditions that are applied may be quite different from those met in soil or be only valid if these happen to coincide. The effect of temperature can be given as an example. PONCHET (1966) reports that at temperatures below 12°C the biological balance during wheat seed germination in soil shifts in favour of the pathogens *Fusarium nivale* and *Septoria nodorum*, at higher temperatures the growth of the plantlets and the development of the saprophytes *Epicoccum* and *Alternaria* contributing to their natural protection is favoured. This shows that the protection offered by the saprophytes may also shift with conditions, a reason for care with methods that would try to incalculate them. In section 5.8 a distinction was also made between pathogen masking and seedling protecting effects. The question raised by HUBER & WATSON (1966) about the validity of agar plate inhibition tests for determining antagonism between soil-microorganism may also have some relevance to comparisons between antagonism observed in the agar method and field performance.

The rather high infection percentages sometimes obtained by the use of more or less selective methods can perhaps be corrected as suggested in chapter 6. In this chapter concepts of inoculum potential and threshold level of infection were introduced into seed health testing. Introducing a correction for inoculum potential (severity of infection) e.g. by the use of a selected standardized pretreatment, could meet the objections against the elimination of such factors as host vigour and antagonism. The higher the inoculum potential the better will be the chances for the pathogen to overcome these factors. If the method is corrected by the use of a pretreatment this should be easy to apply and well standardized.

NaClO pretreatment seems satisfactory at present, but other superior chemical, physical or mechanical treatments may be developed. It is moreover useful that their severity can be dosaged. This would permit selection of the level giving the best correlation with field experiments. In the present work this could not be investigated any more. As discussed in chapter 6, the site of infection and depth of penetration are likely to be intimately related with inoculum potential. In case of infections that are mainly superficial and yet important surface treatments should of course not be used.

Pretreatments should be selected of a severity that indicates minimum inoculum potentials sufficient for causing disease development under natural conditions conducive for disease. Such levels could be selected by comparisons with

more pathogenic methods such as soil tests. In such comparisons one should make sure that the seed-borne pathogen does not happen to be of a less virulent strain. The same applies to the use of a highly resistant host cultivar. Their use would give a false idea of the importance of the risk involved if applied to other seed lots.

In chapter 7 we already mentioned that the prediction of field performance from seed health data can be regarded as very difficult or even completely impossible for most seed-borne infections. The reasons were already discussed in that chapter, in which it was also pointed out that the main importance and danger lies in the epidemiological role of the infections and in the introduction of the pathogen or strains thereof into new areas or fields.

Seed health data should therefore be regarded as only indicating a certain risk factor. The amount of risk depends on the pathogen, on the quantity of infection and on field conditions. For the influence of the region of sowing, NEERGAARD (1961), gives the example of *Phoma lingam* in cabbage seed. If infected seed is sown in Denmark, it causes little harm, but it may cause large losses if sown in warmer countries. The quantity of the infection that can be tolerated differs, consequently, from country to country for each infection and should be reflected in the tolerance levels set.

If such tolerance levels are fixed they should indicate the method to which they apply (cf. NEERGAARD, 1962). This is important as we have met instances in this paper of one method giving higher infection percentages than another one. If used in this way such tolerances would also provide a correction for the method used.

The preceding discussion suggests that further research along the lines started by the author, may lead to the development of simple, accurate, rapid and dependable methods needed for international trade. Often these will be selective methods, so that for a single kind of seed that may contain several pathogens, more than one method would be required. The latter is, however, also the case for many of the less satisfactory methods now being used (cf. 3 blotter methods for 3 groups of cereal seed infections). It is also clear that these are most likely to be methods giving maximum ('absolute') results, which may have to be corrected in a standardized way for inoculum potential.

In the author's opinion such an attitude would satisfy both those who demand methods giving maximum results (e.g. ANSELME, 1962; MALONE & MUSKETT, 1967) as well as those demanding methods most closely predicting field performance (DE TEMPE, 1963b, 1964c). The latter demand would also be met by the use of tolerance levels adjusted to such methods, correcting the method

used for local agricultural conditions in the area of sowing. Further research may provide additional or improved correction factors.

ACKNOWLEDGEMENTS

The present work was made possible by a grant from the U.S. Dept. of Agriculture, for which the author wishes to express his gratitude. He considers himself greatly indebted to dr. J. DE TEMPE, the head of the Department of Seed Health Testing of the Government Seed Testing Station at Wageningen, under whose direction the work was carried out. His sincere thanks are furthermore due to dr. A. F. SCHOOREL, the director of the station for his hospitality and encouraging interest and to dr. A. J. P. OORT, professor of Phytopathology at the Agricultural University at Wageningen, for his general guidance, stimulation and advice. The author is also very greatful to his own assistants, those of dr. J. DE TEMPE and the entire personnel of the station for their help. The regional laboratories of the Certifying Agency for Agricultural Seeds as well as foreign collegues should be thanked for their help in obtaining interesting seed samples.

SUMMARY

The purpose of this study was to investigate the factors affecting the outcome of incubation methods used to test seeds for the presence of fungal pathogens. The reason for starting such an investigation was the poor reproducibility of the results of those methods.

Chapter 3 starts with the observation that for a number of infections lower percentages are found on blotters with a relatively high moisture content than on blotters with a lower moisture content. This so-called 'wet blotter effect' (WBE) was found to be related to the speed of water uptake by the seeds; if the water uptake is relatively slow the effect does not show, whereas in the case of more rapid water absorption the effect can be noted. If the rate of water uptake of seeds without WBE was increased by presoaking them in water, a similar decrease was noted ('soak effect' or SE). WBE and SE could both be nullified by the addition of antibacterial antibiotics, and so were attributed to antagonism by saprophytic seed-borne bacteria.

Factors stimulating bacterial development (higher temperature and pH, addition of nutrients or bacteria) were found to increase WBE and SE; factors stimulating fungi more than bacteria decreased or eliminated it. If one of both groups (bacteria or fungi) obtains the initial lead it will dominate and reduce the expression of the other group.

Elimination of bacterial antagonism results in higher percentages of both pathogenic and saprohytic fungi found. The use of antibiotics eliminates bacterial antagonism as a source of variation.

Bacterial antagonism in the agar method is often less important due to low pH of the medium. This offers an explanation for the higher agar than blotter percentages found for such an infection as *Botrytis cinerea* in flax seed. The hypochlorite pretreatment customary for the testing of most seeds by the agar method also restricts bacterial antagonism. Bacterial antagonism in agar tests may not only lower infection percentages found but also change the colony appearance. The addition of a small quantity of an antibiotic to the agar medium eliminates it altogether.

The importance of the WBE and SE may vary with the sample, due to differences in the bacterial flora of the seeds. Plating soaked seeds on agar media yields hardly any pathogen, but agar media with antibiotics which were routinely used in all further work, more or less nullify the previous SE. Soaking in terramycin solution often gave increased percentages of the pathogen.

The same factors stimulating bacterial antagonism in blotter tests also favour it in sand and soil.

Chapter 4 starts with the observation that in case of *Phoma valerianellae* infection of corn salad seeds blotter tests with 2,4-D gave remarkably higher percentages than normal blotter tests. This led to the realization of the importance of the factor 'host vigour' in incubation methods. Various other treatments such as water soak, exhaustion, mutilation and freezing gave similar results. The reduction (2,4-D), elimination (freezing) or circumvention (agar method) of host vigour often lead to higher percentages being found for seed-borne infections. It also offers an explanation for the difference between blotter and agar test results for some infections. Reduction of the host vigour causes a shift from symptoms of the disease to signs of the pathogen to be looked for at inspection.

The freezing of pre-incubated seed followed by a post-freezing incubation period (freezing method) was found to be promising for a number of infections. For some infections (e.g. *Helminthosporium* spp.) it simplifies inspection, no seedling structures being produced. Provided bacterial development is checked by means of antibiotics, fungi grow easier and more abundantly, probably due to increased leachage of nutrients from seeds. Fungal infections can therefore be more easily recognized and identified, and higher infection percentages may be found. Some seeds which are rich in easily available nutrients (e.g. wheat) must be protected from air-borne contamination. In the freezing method the trays with paper can be replaced by plastic sheets with depressions filled with liquid (containing e.g. antibiotics) for the seeds.

In chapter 5 it is observed that interfungal antagonism becomes more prominent when bacterial antagonism and host vigour are either not important or have been eliminated. It is consequently especially a problem in the agar method. Interfungal antagonism is influenced by incubation conditions, e.g. temperature. Its reduction by the use of hypochlorite pretreatment is rather non-selective, eliminating also part of the pathogen, although it may be all right for such deep-seated infections as *Ascochyta* spp. in pea and *Phoma betae* in beet seeds.

More selective is the 'dry heat' pretreatment advocated by MALONE (1962) for *H. avenae* in oat seeds tested by the agar method. Its use was extended to other *Helminthosporium* spp. in oat and barley seeds but has the disadvantage of increased *Mucor* development in subsequent agar tests. In blotter tests with dry heat pretreatment *Mucor* presented no problem, but the results were not superior to those of the freezing method.

A highly selective method is the use of peptone-PCNB agar for *Fusarium* spp. In this method neither bacterial and fungal antagonism nor host vigour play a role, so that very high infection percentages may be obtained. Tests carried out by this method are easy to prepare and to inspect.

Another way to combat interfungal antagonism is the use of growth-restricting agents, such as oxgall, in agar media. This offered good prospects for the slow growing *Septoria nodorum* in wheat seed, which is otherwise easily suppressed by other fungi. The use of oxgall eliminates the need of surface disinfection which is an advantage as light infections of *S. nodorum* can be important.

Chapter 6 deals with measurement of the severity of infection. Very light *Fusarium* infections of single seeds may be considered to be of little consequence. Their elimination by hypochlorite pretreatment can therefore be accepted and may be used as a correction of the very sensitive peptone-PCNB agar method. This led to considerations on the importance of inoculum potential and threshold level of infection. Seed health data are usually given in terms of percentage of infected seeds. Possible ways to correct them for inoculum potential were indicated, such as the use of pretreatments of standardized strength. This could perhaps compensate for the elimination of such factors as antagonism and host vigour.

Chapter 7 describes experiments in which a large number of wheat seed samples containing *Fusarium* spp. and *Septoria nodorum* were tested in various ways. The results were then compared and correlation coefficients between them were computed. Although the latter were low, they were generally significant. The correlations of germination capacity with soil emergence decreased with temperature due to the presence of *F. nivale*. This pathogen only reduces emergence at lower temperatures, being of no consequence at higher ones. *S. nodorum* did not significantly reduce emergence in the samples used, although at lower temperatures it caused more abnormal seedlings to be produced. *S. nodorum* was always highly correlated with coleoptyle lesions.

The blotter test for *Fusarium* spp. was shown to be very unreliable. The symptoms are hard to judge so that errors of judgment are easily made the presence of the different *Fusarium* spp. is not equally well indicated and other fungi e.g. *S. nodorum* may interfere by producing the same symptoms. The blotter test for *S. nodorum* is only good for a number of cultivars that produce the characteristic protuberance symptoms. For other cultivars the symptoms to be used are highly unreliable, especially if *Fusarium* spp. are present, which usually is the case. The agar method is therefore to be preferred, preferably without surface disinfection, but with e.g. oxgall added to the medium.

It is suggested that due to the complex nature of the factors involved, which are moreover unpredictable the usefulness of correlation coefficients for seed-borne infections is very limited. Their main use may be in the comparison of various methods with seed-

ling performance in conditioned soil tests.

In chapter 8 it is concluded from the preceding that the incubation conditions applied in seed health testsdo not only affect the expression of the pathogen in a direct way. They also affect the pathogen indirectly via the seed biotope (seed microflora and host seed or seedling). The seed biotope varies with the sample so that the most important source of variation is presented by the ecology of the germinating seed itself. Elimination and circumvention of these biotic factors, which also decreases the chances of errors of judgment, seem to offer the best prospects for the development of simple, cheap, fast and reproducible methods.

Application of correction factors for inoculum potential may be important for a number of infections (e.g. *Fusarium* spp. in cereal seeds). Tolerances if used may be regarded as a correction for local agricultural conditions and should be based on the methods used for testing.

SAMENVATTING

Ecologische aspecten van het gezondheidsonderzoek van zaaizaden

Het onderzoek betrof de achtergronden van de bepaling van pathogene schimmelinfecties van zaaizaden d.m.v. incubatiemethoden. Aanleiding tot dit onderzoek was de slechte reproduceerbaarheid van deze methoden.

In hoofdstuk 3 wordt geconstateerd, dat voor bepaalde zaadinfecties op filtreerpapier met een relatief hoog vochtgehalte, lagere besmettingspercentages worden gevonden dan op filtreerpapier met een lager vochtgehalte. Dit zogenaamde 'wet blotter effect' ('WBE') bleek samen te hangen met de snelheid van wateropname door de zaden; zaden welke water relatief langzaam opnemen vertonen het effect niet, terwijl in geval van een snellere wateropname het effect aanwezig is. Indien de snelheid van wateropname door voorweken wordt verhoogd verkrijgt men een soortgelijke verlaging, het 'soak effect' ('SE'). 'WBE' en 'SE' konden beide worden opgeheven door toevoeging van antibacteriële antibiotica, en werden daarom toegeschreven aan antagonisme door saprofytische op het zaad aanwezige bacteriën.

Factoren die bacteriegroei bevorderen (hoge temperatuur en pH, toevoeging van voedingsstoffen of van de bacteriën zelf) versterkten het 'WBE'en'SE'. Omstandigheden die voor de groei van schimmels gunstiger zijn dan voor die van bacteriën, verminderden of elimineerden deze effecten. Na uitschakeling van bacterieel antagonisme vindt men dus hogere percentages voor pathogene en ook voor saprofytische schimmels.

In de agarmethode is bacterieel antagonisme vaak minder belangrijk vanwege de relatief lage pH en lage oppervlaktevochtigheid van het medium. Dit geeft een verklaring voor het feit, dat de agarmethode veelal hogere percentages oplevert dan de filtreerpapiermethode, bijv. voor *Botrytis cinerea* in vlaszaad. De voorbehandeling met hypochlorietoplossing, gebruikelijk voor het toetsen van de meeste zaadsoorten met behulp van de agarmethode, beperkt het bacterieel antagonisme eveneens. Toch worden

door bacterieel antagonisme ook in de agarmethode verlaagde infectiepercentages gevonden. Tevens kan het uiterlijk van de kolonies veranderen. De toevoeging van een kleine hoeveelheid antibioticum aan het agarmedium schakelt de bacteriële activiteit geheel uit.

Het 'WBE' resp. het 'SE' variëren met het monster, vanwege verschillen in de bacterieflora van de zaden. Het uitleggen van geweekte zaden op agarmedia zonder antibiotica toont nauwelijks pathogene infectie aan, maar met antibiotica in de agar zoals dit in alle verdere werk regelmatig werd gebruikt, werd de invloed van het voorweken ('SE') praktisch ongedaan gemaakt. Voorweken in een terramycine-oplossing resulteerde dikwijls in een hoger infectiepercentage.

Dezelfde factoren welke bacterieel antagonisme in de filtreerpapiermethode stimuleren, bevorderen het eveneens in zand en grond.

Hoofdstuk 4 begint met het feit, dat de 2,4-D filtreerpapierproef voor *Phoma valerianellae*-infectie van veldslazaad aanzienlijk hogere percentages gaf dan de normale filtreerpapiermethode. Dit vestigde de aandacht op de factor gastheerweerstand ('host vigour') in de incubatiemethoden. Verschillende andere behandelingen zoals voorweken in water, uitputting, beschadiging en bevriezing, gaven soortgelijke resultaten. De vermindering (2,4-D), uitschakeling (bevriezen) of vermijding (agarmethode) van de gastheerweerstand leidde dikwijls tot het vinden van hogere percentages. Dit draagt ook bij tot het verschil in resultaat van de filtreerpapier- en agarmethode voor sommige infecties. Reductie van de gastheerweerstand veroorzaakt een verschuiving van ziektesymptomen naar schimmelgroei als blijk van aanwezigheid van de infectie.

Het bevriezen van kiemende zaden (na voorincubatie), gevolgd door verdere incubatie van de bevroren zaden, wordt veelbelovend geacht voor een aantal infecties. Deze vriesmethode vereenvoudigt de inspectie, omdat geen kiemplant wordt gevormd. Vooral als tevens de bacterieontwikkeling wordt onderdrukt d.m.v. antibiotica, groeien de schimmels gemakkelijker en weelderiger, waarschijnlijk t.g.v. toegenomen diffusie van voedingsstoffen uit de zaden. Men kan de infecties met meer zekerheid herkennen en vindt er hogere percentages van. Als de gastheerweerstand een rol speelt, voert uitschakeling ervan soms tot het vinden van veel hogere infectiepercentages. Zaden die rijk aan direct beschikbare voedingsstoffen zijn (bijv. tarwe), moeten tegen infecties vanuit de lucht (*Mucor* spp.) worden beschermd. In de vriesmethode kunnen de bakjes met filtreerpapier worden vervangen door dun plastic met ingeperste kuiltjes, gevuld met vloeistof (waarin antibiotica) voor de zaden.

In hoofstuk 5 wordt opgemerkt, dat antagonisme tussen schimmels onderling meer op de voorgrond gaat treden wanneer bacterieel antagonisme en gastheerweerstand niet belangrijk zijn, of worden uitgeschakeld. Het is daarom vooral een probleem in de agarmethode.

Antagonisme tussen schimmels wordt beïnvloed door de incubatieomstandigheden, zoals de temperatuur. Vermindering van dit antagonisme door voorbehandeling met een hypochlorietoplossing is weinig selectief, omdat het ook een deel van het pathogeen uitschakelt. Het is echter waarschijnlijk zonder bezwaar bruikbaar voor dieper zittende infecties zoals *Ascochyta* spp. in erwte-, en *Phoma betae* in bietezaden.

Het droog verhitten, toegepast door MALONE (1962) voor *H. avenae* in haverzaden

voor toetsing met de agarmethode, is selectiever. Het bleek ook bruikbaar voor andere *Helminthosporium* spp. in haver- en gerstzaden. Deze behandeling heeft het nadeel van een toegenomen *Mucor* ontwikkeling in de daaropvolgende agarproef. In de filtreerpapierproef met zaden die verhit geweest zijn, was *Mucor* geen probleem, maar de resultaten waren niet beter dan die van de vriesmethode.

Een zeer selectieve methode is het gebruik van pepton-PCNB agar voor *Fusarium* spp. In deze methode spelen noch bacterieel- en schimmelantagonisme, noch gastheerweerstand een rol zodat veel hogere infectiepercentages worden aangetoond.

Een andere manier om antagonisme tussen schimmels onderling tegen te gaan is het gebruik van groeiremmende stoffen, zoals ossegal, in agarmedia. Dit biedt goede mogelijkheden voor de langzaam groeiende *Septoria nodorum* in tarwezaad, die anders door andere schimmels gemakkelijk wordt onderdrukt. Het gebruik van ossegal maakt oppervlakkige ontsmetting overbodig, wat een groot voordeel is omdat lichte infecties van *S. nodorum* belangrijk kunnen zijn.

Hoofdstuk 6 behandelt het meten van hevigheid der infectie. Zeer lichte *Fusarium*-infecties van afzonderlijke zaden kunnen als van gering belang worden beschouwd. Hun uitschakeling d.m.v. voorbehandeling met hypochloriet kan daarom worden aanvaard en gebruikt als een correctie voor de zeer gevoelige pepton-PCNB agarmethode. Dit leidde tot beschouwingen over het belang van inoculum-potentiaal en drempelwaarde van de infectie. De resultaten van gezondheidsonderzoek worden gewoonlijk gegeven als percentages geïnfecteerd zaad. Mogelijkheden ze te corrigeren voor inoculum-potentiaal worden aangegeven, zoals het gebruik van een gestandaardiseerde licht ontsmettende voorbehandeling. Dit zou misschien de uitschakeling van factoren als antagonisme en gastheerweerstand kunnen compenseren.

In hoofdstuk 7 worden proeven beschreven in welke tarwezaadmonsters met *Fusarium* spp. en *Septoria nodorum* op diverse wijzen werden getoetst. De resultaten zijn vergeleken, en correlatiecoëfficiënten berekend. Hoewel deze meestal tamelijk laag bleken, waren ze nochtans veelal significant. De correlaties tussen kiemkracht en opkomst in grond verminderden bij verlaging van de temperatuur vanwege de aanwezigheid van *F. nivale*. Dit pathogeen vermindert de opkomst bij lagere temparaturen, maar is van geen betekenis bij hogere temperaturen. *S. nodorum* reduceerde de opkomst van de gebruikte monsters niet, hoewel hij meer abnormale kiemplanten veroorzaakte bij lagere temperaturen. *S. nodorum* was altijd sterk gecorreleerd met coleoptiel-lesies in grondproeven.

De filtreerpapiermethode voor *Fusarium* spp. werd zeer onbetrouwbaar bevonden. De symptomen zijn moeilijk te beoordelen zodat gemakkelijk beoordelingsfouten kunnen worden gemaakt, de verschillende *Fusarium* spp. worden niet alle even goed aangetoond en andere schimmels, zoals bijv. *Septoria nodorum*, kunnen dezelfde symptomen geven.

De filtreerpapiermethode voor *S. nodorum* is slechts geschikt voor een aantal cultivars welke het karakteristieke wratjessymptoom geven. Voor andere cultivars zijn de kiemplantsymptomen, die gebruikt zouden kunnen worden, zeer onbetrouwbaar, in het bijzonder als *Fusarium* spp. aanwezig zijn, wat gewoonlijk het geval is. De agarmethode is daarom te prefereren, liefst zonder oppervlakkige desinfectie, maar met

ossegal toegevoegd aan het medium. Het nut van correlatiecoëfficiënten blijkt vooral te liggen in de vergelijking van de uitkomsten van verschillende laboratoriummethoden voor gezondheidsonderzoek met kiemplantontwikkeling in geconditioneerde grondproeven.

In hoofdstuk 8 wordt uit het voorgaande de conclusie getrokken, dat de incubatieomstandigheden, toegepast in het gezondheidsonderzoek van zaaizaden, de manifestatie van het pathogeen niet slechts op een directe wijze beïnvloeden. Zij beïnvloeden het pathogeen ook sterk indirect via het zaadbiotoop (zaad microflora en het kiemende zaad). Het zaadbiotoop varieert met het monster, zodat de belangrijkste bron van variatie is gegeven door de ecologie van het kiemende zaad zelf. Uitschakeling of vermijding van deze biotische factoren biedt vermoedelijk de beste vooruitzichten voor de ontwikkeling van eenvoudige, goedkope, snelle en reproduceerbare methoden.

De toepassing van correctiefactoren voor inoculum-potentiaal zou belangrijk kunnen zijn voor een aantal infecties (bijv. *Fusarium* spp. in graanzaden). Toleranties kunnen worden gebruikt als een correctie voor lokale uitzaai-omstandigheden en moeten betrekking hebben op de methode gebruikt voor het onderzoek.

RÉSUMÉ

Études sur certains aspects écologiques de l'analyse sanitaire des semences

Le but de cette étude a été d'examiner les conditions qui affectent les conclusions qui peuvent être tirées des méthodes d'incubation employées pour détecter la présence des pathogènes fongiques sur les semences. La variabilité des résultats de ces méthodes nous a fait entreprendre cette étude.

Au chapitre 3 on constate que dans certains cas le nombre des graines infectées trouvé est moins élevé à mesure que le papier buvard est plus humide, si on employe la méthode sur papier buvard. Ce phénomène appelé 'wet blotter effect' ('WBE') depend surtout de la nature spécifique de la semence et par des pathogènes associés aux semences. Le 'WBE' depend de la rapidité d'absorbtion d'eau; si l'absorbtion est lente ce phénomène est absent, si l'eau est plus rapidement absorbée l'effet peut être démontré. En accélerant l'absorbtion d'eau par trempage les semences normalement sans 'WBE' montrent un effect pareil ('soak effect' ou 'SE'). Le 'WBE' ainsi que le 'SE' peuvent être anéanties par l'addition de substances antibiotiques actives envers les bactéries, et de ce fait ces phénomènes ont attribués à un antagonisme entre des champignons et des bactéries présent sur la graine.

Il a été constaté que les conditions qui favorisent le développement des bactéries (température, pH, addition de subtances nutritives) augmentent le 'WBE' et le 'SE'. Celles qui favorisent le développement des champignons diminuent ces effects. Si au

début, un de ces groupes (bactéries ou champignons) se trouve dans une situation favorable, il dominera en diminuant l'expression du dernier. L'élimination des bactéries par substances antibiotiques fait qu'on trouve un pourcentage d'infection plus élevé sur les graines. L'emploi des substances antibiotiques élimine l'antagonisme bactérien comme source de variation dans l'analyse sanitaire des semences sur papier buvard.

En employant la méthode sur milieux gélose en pH rélativement bas l'antagonisme bactérien est géneralement moins important que dans la méthode sur papier buvard. Cela s'explique par le fait que pour certaines infections (par examples *Botrytis cinerea* dans les semences du lin) les résultats obtenus avec la méthode sur gélose sont généralement plus élevés que les résultats obtenus sur papier buvard. La désinfection superficielle des graines avec une solution d'hypochlorite de sodium, normalement utilisée pour la plupart des semences avant leur analyse sur des substrats gélosés, réduit également l'antagonisme bactérien. L'antagonisme bactérien dans la méthode sur gélose ne peut pas seulement réduire le nombre de colonies fongiques mais il peut aussi changer leur aspect extérieur. Il est complètement éliminé par l'addition d'une petite quantité de substances antibiotiques au milieu gélose.

L'importance du 'WBE' et du 'SE' varie selon la flore bactérienne des échantillons des semences. L'analyse sanitaire des semences par la méthode sur gélose normal après leur trempage pour 24 heures ou plus dans l'eau entraîne un développement fongique assez réduite à cause d'une croissance luxuriante des bactéries. L'addition de substances antibiotiques au milieu gélose nullifie aussi un tel 'SE'. Dans tous les travaux suivants la substance antibiotique terramycine est toujours ajoutée aux milieux géloses. Le trempage des semences dans des solutions aqueuses de terramycine amène souvent qu'on trouve un plus grand nombre de graines infectées par les analyses subséquentes.

Les mêmes conditions qui stimulent l'antagonisme bactérien sur papier buvard le favorisent également dans des essais et en sable et en terre.

Le chapitre 4 commence par l'observation que la méthode sur papier buvard, avec adjonction de 2,4-D, indique plus de graines de la mache étant infectée par le *Phoma valerianellae* que le même méthode sans adjonction de 2,4-D. Des traitements divers, comme le trempage dans l'eau, l'exténuation, la mutilation et la congélation, produisent des résultats pareils. Cela donna lieu à prendre conscience de l'importance d'élement de la vitalité de la plante hôte ('host vigour'). La réduction (2,4-D), l'élimination (congélation) ou l'évitement (sur gélose) de la vitalité des graines en germination dans l'analyse sanitaire des semences, entraîne souvent qu'on trouve un plus grand nombre de graines infectées. Pour certaines infections il donne aussi une explication sur la différence entre les résultats des analyses obtenus par la méthode sur papier buvard et ceux sur gélose. La réduction de la vitalité des graines en germination amène aussi un changement dans la manifestation de la présence du pathogène des symptômes de la maladie aux structures du pathogène même.

La congélation des semences après une période préliminaire d'incubation (méthode à congélation) promet d'être une bonne méthode pour l'analyse sanitaire d'un nombre d'infections. La dite méthode simplifie l'inspection car il n'y a pas la formation de plantules. Quand le développement des bactéries est inhibé par des substances antibiotiques, les champignons croissent plus facilement et d'une façon plus luxuriante probable-

ment par suite d'une augmentation de la diffusion des substances nutritives par les graines. On peut reconnaître la présence d'une infection avec plus de certitude. Si la vitalité de la plante hôte joue un rôle, son élimination entraîne souvent un pourcentage plus élevé. Les semences qui contiennent beaucoup de substances nutritives facilement disponibles (p. ex. le froment) doivent être protégé contre la contamination apportée par l'air. Dans la méthode à congélation les boîtes avec papier buvard peuvent être remplacées par des lames en plastique à petites cavités remplies d'un liquide (contenant des substances antibiotiques) pour y mettre les graines.

Au chapitre 5 on remarque que l'antagonisme entre champignons (interfongique) monte au premier plan quand l'antagonisme bactérien et la vitalité de la plante hôte ne sont ou bien pas importants, ou ont été éliminés. Par conséquent il est surtout important dans la méthode sur gélose. L'antagonisme interfongique est aussi influencé par les conditions d'incubation (la température p. ex.). Sa réduction par une désinfection superficielle des graines est peu sélective, éliminant aussi une partie du pathogène, bien qu'il peut être acceptable pour les infections qui sont généralement plus profondes, comme les *Ascochyta* spp. dans les semences de pois et la *Phoma betae* dans les semences de betterave.

Le prétraitement des semences dans une étuve en présence d'air de 100°C pendant une heure avant de les déposer à la surface d'un milieu gélosé, appliqué par MALONE (1962) pour découvrir *H. avenae* dans les semences d'avoine, est plus sélectif. Il s'est trouvé aussi utilisable pour d'autres espèces d'*Helminthosporium* dans les semences d'avoine et d'orge. Ce prétraitement a le désavantage d'une augmentation du développement des espèces de *Mucor* dans l'essai subséquent sur gélose. Il se trouva qu'il n'y eut pas de problèmes posés par des Mucorales dans les essais sur papier buvard après ledit prétraitement.

Une méthode hautement sélective est l'utilisation du milieu peptone-PCNB gélosé pour la détection des *Fusarium* spp. Dans cette méthode ni l'antagonisme bactérienne et interfongique, ni la vitalité de la plante hôte jouent un rôle, de sorte qu'on trouve des pourcentages plus élevés de semences infectées par *Fusarium* spp. La préparation et l'inspection dans ladite méthode sont très simples.

Une autre manière à combattre l'antagonisme interfongique est par l'emploi des substances qui restrennent la croissance des colonies fongiques, comme p. ex. l'addition de bile de boeuf aux milieux géloses. Cela offre de bonnes possibilités pour détecter le *Septoria nodorum* sur les semences du blé, parce que ce champignon croît très lentement et il est facilement supprimé par les autres. L'application de la bile de boeuf dans les milieux géloses élimine la nécessité de la désinfection superficielle ce qui est un avantage parce que des infection légères de *S. nodorum* peuvent être d'importance.

Au chapitre 6 le mesurage de la sévérité de l'infection est discuté. Les infections très légères des graines individuelles par des *Fusarium* spp. peuvent être considerées comme ayant peu d'importance. Leur élimination par un prétraitement avec une solution d'-hypochlorite de sodium est acceptable de ce fait et peut être employé comme correction pour la méthode très sensible sur milieu peptone-PCNB gélosé. Il donna lieu aux considérations sur le potentiel d'inoculum ('inoculum potential') et sur le niveau de seuil ('threshold level') d'infection. Les résultats des analyses sanitaires des semences

sont d'ordinaire exprimés comme pourcentage des graines dans lesquelles on a découvert la présence d'un certain pathogène. Des possibilités de les corriger portant sur le potentiel d'inoculum sont indiquées, tel que l'application d'une intensité standardisée. Il pourrait compenser l'élimination de la microflore des semences et de la vitalité de la plante hôte.

Au chapitre 7 on trouve une description des expériences sur diverses méthodes pour la détection des *Fusarium* spp. et le *Septoria nodorum* dans des échantillons des semences du blé. Les résultats furent comparés et leurs coefficients de corrélation calculés. Bien qu'ils soient généralement assez bas, ils sont néanmoins le plus souvent significatifs. La corrélation entre la faculté germinative et l'émergence dans la terre décrût avec la température par suite de la présence du *F. nivale*. Ce pathogène réduit l'émergence en terre seulement à des températures basses (sous 15 °C), mais n'a pas d'importance aux températures plus élevées. Le *S. nodorum* réduisit pas significativement l'émergence pour les échantillons employés, bien qu'il causa la formation de plus de plantules anormales, à basses températures. Il y eut toujours une bonne corrélation entre le présence du *S. nodorum* dans les semences et la formation des lésions sur les coléoptiles des jeunes plantules en terre.

La méthode sur papier buvard pour la détection des *Fusarium* spp. se montra peu sûr. La critique des symptômes est difficile, les différents *Fusarium* spp. ne sont pas détectés aussi bons; tandis que d'autres champignons (p. ex. *S. nodorum*) peuvent produire, eux, des symptômes identiques. La méthode sur papier buvard pour la détection du *S. nodorum* n'est propre qu'à un certain nombre de cultivars donnant des symptômes caractéristiques sur la formation des protubérances. Pour les autres cultivars qui ne donnent pas ledit symptôme on doit recourir aux autres symptômes qui sont trop incertains, surtout dans la présence usuelle des *Fusarium* spp. La méthode sur gélose est préférable de ce fait, surtout celle sur milieu gélosé contenant de la bile de boeuf qui élimine la nécessité d'une infection superficielle.

L'utilisation des coefficients de corrélation pourrait être surtout d'importance pour la comparaison des résultats au laboratoire avec le développement des jeunes plantules dans des essais en sol conditionné.

Au chapitre 8 on tire la conclusion que les conditions de l'incubation appliquée à l'analyse sanitaire des semences, n'affectent pas seulement la pathogène en manière directe. Ils l'affectent aussi fortement par la voie du biotope de la semence (l'ensemble de la graine en germination et de sa microflore). Ce biotope varie selon l'échantillon, de sorte que la source de la variation la plus importante est donnée par l'écologie de la graine elle-même en germination. L' élimination, ou l'évitement de ces élements biotiques donne à ce qu'il paraît les meilleures perspectives pour le développement des méthodes simples, bon marché, rapides et réproduisibles.

L'application des corrections pour le potentiel d'inoculum pourrait être importante pour un certain nombre d'infections (p. ex. des *Fusarium* spp. dans les semences des céréales). Les niveaux de tolérances peuvent être appliqués comme une correction pour les conditions locales et doivent être ajustés à la méthode d'essai utilisée.

Zusammenfassung

Oekologische Aspekte der Gesundheitsprüfung von Samen

Der Zweck der hier veröffentlichten Untersuchungen war den Einfluss der Umweltfaktoren, auf die Ergebnisse der Inkubationsmethoden für die Prüfung von Samen auf die Anwesenheit phytopathogener Pilze, besser kennenzulernen. Der Anlass für diese Forschung war die schlechte Standardisierung solcher Methoden.

Im Kapitel 3 wird festgestellt, dass bei bestimmten Sameninfektionen niedrigere Prozentsätze von infizierten Samen auf Fliesspapier mit einem verhältnismässig hohen Wassergehalt gefunden werden, wie auf Fliesspapier mit einer niedrigen Substratfeuchtigkeit. Dieser sogenannte 'wet blotter effect' ('WBE') schien mit der Geschwindigkeit der Wasseraufnahme durch die Samen verbunden zu sein; Samen welche Wasser verhältnismässig langsam aufnehmen, haben diesen Effekt nicht gezeigt, während im Fall einer schnelleren Wasseraufnahme der Effekt vorhanden war. Wenn die Geschwindigkeit der Wasseraufnahme für Samen ohne 'WBE' durch das Vorweichen in Wasser erhöht wird, gibt es eine ähnliche Herabsetzung ('soak effect' oder 'SE'). Sowohl 'WBE' wie 'SE' konnten durch Hinzufügung von antibakteriellen Antibiotika eliminiert werden, und wurden deshalb dem Antagonismus von saprophytischen samenbegeleitenden Bakterien zugeschrieben. Faktoren, welche das Bakterienwachstum fördern (hohe Temperatur und pH, Zusatz von Nährstoffen oder von Bakterien selbst), erhöhten 'WBE' und 'SE'. Umstände, welche für das Wachstum von Pilzen günstiger sind wie für das Wachstum von Bakterien, haben diese Effekte vermindert oder eliminiert.
Nach Ausschaltung des bakteriellen Antagonismus findet man deshalb höhere Prozentsätze von pathogenen und auch von saprophytischen Pilzen.

Bei der Agarplattenmethode ist der bakterielle Antagonismus oft weniger wichtig wegen des verhältnismässig niedrigen pH Wertes und der geringen Oberflächenfeuchtigkeit des Substrats. Dies ist eine Erklärung dafür, dass die Agarmethode vielfach höhere Prozentsätze ergibt wie die Fliesspapiermethode, z.B. für *Botrytis cinerea* in Leinsamen. Die Vorbehandlung mit Natriumhypochlorit, die bei der Prüfung der meisten Sämereien mittels der Agarmethode gebräuchlich ist, schränkt ebenfalls den bakteriellen Antagonismus ein. Dennoch werden auch bei der Agarmethode die gefundenen Infektions-Prozentsätze durch bakteriellen Antagonismus herabgesetzt. Zugleich kann sich das Pilzwachstum in einer ganz anderen Form zeigen. Durch den Zusatz einer kleinen Menge des Antibiotikums wird die Aktivität der Bakterien gänzlich ausgeschaltet.

'WBE' bzw. 'SE' zeigen von der Probe, auch in Abhängigkeit von der verschiedenen Bakterieflora, Unterschiede. Das Auslegen von in Wasser geweichten Samen auf den Agarboden ohne Antibiotikum zeigt gerade keine Infektion. Bei der Prüfung der geweichten Samen auf Agarboden mit dem Zusatz von Antibiotikum wurde dagegen nahezu kein 'SE' gefunden. Das Vorweichen in einer Terramycinlösung ergab oft einen höheren Prozentsatz.

Dieselben Faktoren, welche den Bakterienantagonismus bei der Fliesspapiermethode stimulieren, fördern diesen gleichfalls in Versuchen in Sand und Boden.

Das Kapitel 4 beginnt mit der Beobachtung, dass mittels der Fliesspapiermethode mit dem Zusatz von 2,4-D für *Phoma valerianellae* in Feldsalatsamen, ein höherer Prozentsatz der Infektion gefunden wurde wie mit der normalen Fliesspapiermethode. Dies lenkte die Aufmerksamkeit auf den Faktor der Lebenskraft des Wirtes ('Host-vigour') in Inkubationsmethoden. Mehrere andere Behandlungen, wie Vorweichen in Wasser, Erschöpfung ,Verletzung und Gefrieren ergaben ähnliche Ergebnisse. Die Herabsetzung (2,4-D), die Ausschaltung (Totfrieren) oder die Vermeidung (Agarmethode) von der Vitalität des Wirtes führte oft dazu, dass höhere Infektions-Prozentsätze gefunden wurden. Bei bestimmten Infektionen kann dies eine Erklärung für die Differenz zwischen den Resultaten der Fliesspapier- und der Agarmethode sein. Die Herabsetzung der Vitalität des Wirtes hat auch eine Verschiebung der Krankheitssymptome durch Pilzstrukturen als Zeichen für die Anwesenheit der Infektion zur Folge.

Das Einfrieren nach einer bestimmten Vorinkubationszeit gefolgt durch eine Nachinkubation (Gefriermethode) scheint eine gute Methode für bestimmte Infektionen zu sein. Für manche Infektionen, z.B. *Helminthosporium* spp. vereinfacht es die Inspektion, da keine Keimlinge gebildet werden. Insbesondere, wenn zugleich die Entwicklung der Bakterien durch den Zusatz von Antibiotikum unterdrückt wird, wachsen die Pilze – wahrscheinlich infolge der Zunahme der Nährstoffdiffusion aus den Samen – leichter. Man kann die Infektion mit mehr Sicherheit bestimmen und von dieser höhere Prozentsätze finden. Wenn die Lebenskraft des Wirtes eine Rolle spielt, führt ihre Ausschaltung bisweilen zu viel höheren Infektions-Prozentsätzen. Samen, welche reich an unmittelbar verfügbaren Nährstoffen sind (z.B. Weizen), müssen gegen Luftinfektionen *(Mucor)* geschützt werden. Bei der Gefriermethode können die Träger mit Fliesspapier durch dünnen Plaststoff mit eingepressten Külchen, gefüllt mit Flüssigkeit (mit Antibiotikum) für die Samen, ersetzt werden.

Im Kapitel 5 ist die Beobachtung enthalten, dass wenn der Bakterienantagonismus und die Lebenskraft des Wirtes nicht wichtig oder ausgeschaltet sind, der Antagonismus zwischen Pilzen mehr in den Vordergrund tritt. Dies ist daher besonders in der Agarmethode ein Problem. Der Antagonismus zwischen Pilzen wird auch durch solche Inkubationsumstände beeinflusst, wie die Temperatur. Die Herabsetzung von diesem Antagonismus durch Vorbehandlung mit Natriumhypochlorit ist nicht selektiv, da sie auch einen Teil des Pathogens ausschaltet. Es ist aber wahrscheinlich keine Beschwerde für tiefer lokalisierten Infektionen, wie *Ascochyta* spp. in Erbsen- und *Phoma betae* in Rübensamen.

Die Vorbehandlung der Samen in einem Trockenschrank bei 100°C, die MALONE (1962) für *H. avenae* in Hafersamen vor der Prüfung mit der Agarmethode angewendet hat, ist selektiver. Es zeigte sich auch, dass sie für andere *Helminthosporium* spp. in Samen von Hafer und Gerste angewendet werden kann. Diese Vorbehandlung hat den Nachteil, dass sie gleichfalls zu einer Zunahme der Entwicklung von *Mucor* in der darauffolgenden Prüfung auf Agarboden führt. Bei der Fleisspapiermethode mit vorher erhitzten Samen verursachte *Mucor* keine Schwierigkeiten, die Resultate waren jedoch nicht besser, wie jene der Gefriermethode.

Eine sehr trennscharfe Methode ist die Anwendung von Pepton-PCNB Agar für *Fusarium* spp. In dieser Methode spielt noch der Bakterien- und Pilzantagonismus, noch

die Lebenskraft des Wirtes eine Rolle, so dass viel höhere Infektions-Prozentsätze aufgezeigt werden können.

Eine andere Weise für das Einschränken des Antagonismus zwischen Pilzen ist die Anwendung von wachstumhemmenden Stoffen wie Ochsengalle, zum Agarnährboden. Dies bietet gute Perspektiven für die Bestimmung der langsam wachsenden *Septoria nodorum* in Weizensamen. Dieser Pilz wird sonst von anderen Pilzen leicht unterdrückt. Die Verwendung von Ochsengalle macht die Oberflächen-Desinfektion überflüssig, was ein grosser Vorteil ist, da leichte Infektionen mit *S. nodorum* wichtig sein können.

Im Kapitel 6 wird das Messen des Befallsgrades besprochen. Sehr leichte *Fusarium*-Infektionen einzelner Samen können als unwesentlich betrachtet werden. Ihre Ausschaltung durch die Vorbehandlung mit Natriumhypochlorit kann deshalb akzeptiert und als eine Korrektion für die sehr empfindliche Pepton-PCNB Agarmethode angewendet werden. Dies führte zu Betrachtungen über die Bedeutung des Inokulum-Potentials und Schwellwertes der Infektion. Die Resultate der Gesundheitsprüfungen von Samen werden gewöhnlich in Prozenten der infizierten Samen dargestellt.

Möglichkeiten sind angegeben um sie für das Inokulum-Potential zu korrigieren, ebenso auch für die Anwendung einer standardisierten Vorbehandlung. Dies könnte vielleicht auch ergänzt werden für die Ausschaltung solcher Faktoren, wie der Antagonismus und die Vitalität des Wirtes.

Im Kapitel 7 werden Versuche beschrieben, in Welchen Weizensamen mit *Fusarium* spp. und *Septoria nodorum* auf mehrer Weise geprüft wurden. Die Resultate werden verglichen und Korrelationskoeffiziente berechnet. Obwohl diese meistens ziemlich niedrig waren, sind sie jedoch vielfach signifikant. Die Korrelation zwischen Keimkraft und Aufgang im Boden wurde bei niedrigen Temperaturen durch die Anwesenheit von *F. nivale* vermindert.

Dieser Krankheitserreger verringert den Aufgang bei niedrigen Temperaturen, ist jedoch von keiner Bedeutung bei höheren Temperaturen. *S. nodorum* hat den Aufgang der Saatgutproben nicht herabgesetzt, obwohl es bei niedrigen Temperaturen mehr abnormale Keimlinge verursachte. Es zeigte sich immer eine gute Korrelation zwischen dem Prozentsatz von *S. nodorum* in den Samen und den braunen nekrotischen Flecken auf den Koleoptilen der Keimlinge in Bodenversuchen.

Die Fliesspapiermethode erwies sich für *Fusarium* spp. als sehr unzuverlässig. Die Symptome sind nicht nur schwier zu beurteilen, so dass leicht Beurteilungsfehler gemacht werden, aber liefern auch keinen gleich guten Nachweis für *Fusarium* spp., da auch andere Pilze, z.B. *Septoria nodorum*, die gleichen Symptomen hervorrufen können.

Die Fliesspapiermethode für *S. nodorum* ist nur für solche Kultivare geeignet, die charakteristische Vorwölbungsymptome zeigen. Für andere Kultivare sind die Keimlingsymptome welche gebraucht werden können sehr unzuverlässig, besonders dann, wenn wie gewöhnlich auch Arten von *Fusarium* spp. anwesend sind.

Der Agarplattenmethode muss deshalb wo möglich ohne oberflächlicher Desinfektion der Vorzug gegeben werden, jedoch mit dem Zusatz von Ochsengalle zum Agarboden. Man kann annehmen, das wegen der komplizierten Natur der beteiligten Faktoren, welche überdies nicht voraussehbar sind, Korrelationskoeffizienten beim Studium von Sameninfektionen nur in beschränktem Masse anwendbar sind. Der Nutz-

effekt von Korrelationskoeffizienten zeigt sich besonderes in der Vergleichung verschiedener Labormethoden für die Keimlingentwicklung in Bodenversuchen.

Im Kapitel 8 wird aus dem Vorhergehenden der Schluss gezogen, dass die für die Gesundheitsprüfung von Samen angewandten Inkubationsverhältnisse die Manifestation des Pathogens nicht nur auf direkter Weise, sondern das Pathogen auch indirekt durch die Oekologie des keimenden Samen selbst beeinflussen. Die Ausschaltung oder Vermeidung dieser biotischen Faktoren bietet wahrscheinlich die besten Aussichten für die Entwicklung von einfachen, billigen, schnellen und gut standardisierbaren Methoden.

Die Anwendung von Korrektionsfaktoren für das Inokulum-Potential könnte für einige Infektionen (z.B. *Fusarium* spp. in Getreidesamen) wichtig sein. Toleranzen können angewendet werden, wie eine Korrektion für lokalen Aussaatverhältnisse und müssen auf die für die Prüfung angewendete Methode bezogen werden.

REFERENCES

ANONYMOUS (1966): International rules for testing seeds. Proc. int. Seed Test. Assoc. 32: 1–152.

ANSELEME, C. (1962): Introduction à la discussion. Proc. int. Seed Test. Assoc. 27: 879–888.

ANSELME, C., F. JAILLOUX & R. CHAMPION (1966): Méthodes d'analyses sanitaire des semences de lin. Proc. int. Seed Test. Assoc. 31: 157–167

ARNY, D. C. & C. LEBEN (1954): Control of several small grain diseases by the water soak method. Phytopathology 44: 481

ASH, C. G. & O. N. ALLEN (1948): A comparison of methods recommended for the surface sterilization of leguminous seeds. Proc. Soil Sci. Soc. Am. 13: 279–283.

BAKEL, J. J. M. VAN & J. DE KRAKER (1962): Het optreden en de bestrijding van vallers (*Phoma lingam* (Tode) Desm.) in sluitkool. J. versl. Proefstn. Groenteteelt volle Grond Ned. No 13 (18 pp).

BAKER, K. & S. H. SMITH (1966): Dynamics of seed transmission of plant pathogens. A. Rev. Phytopath. 4: 311–334.

BECKER, G. J. F. (1963): Glume blotch of wheat caused by *Leptosphaeria nodorum* Müller. Tech. Ber. Sticht. Ned. Graan-Cent. No. 11 (36 pp.)

BERESOVA, J. P. (1939): A bacterial method for the control of fungous diseases of agricultural plants. I. Seed 'bacterization' as a means for the control of flax-diseases. Mikrobiologiya 8: 186–197 (Rev. appl. Mycol. 20: 534–535)

BEVER, W. M. (1948): Effect of 2,4-D in culture medium on the growth of three pathogenic fungi. Phytopathology 38: 1038

BOSE, S. R. (1947): Hereditary (seed-borne) symbiosis in *Casuarina equisetifolia* Forst. Nature, Lond. 159: 512–514

BOSE, S. R. (1956): *Alternaria* within the pericarp of wheat seed. Nature, Lond. 178: 640–641

BOWEN, G. D. (1961): The toxicity of legume seed diffusates toward *Rhizobia* and other bacteria. Pl. Soil 15: 155–165

BRÖNNIMANN, A. (1968): Zur Kenntnis von *Septoria nodorum* Berk., dem Erreger der Spelzenbräune und einer Blattdürre des Weizens. Phytopath. Z. 61: 101–146.

BUTLER, F. C. (1961): Root and foot rot diseases of wheat. Dept. Agric. N.S.W. Sci. Bull. 77 (98 pp.)

CHRISTENSEN, C. M. (1957): Deterioration of stored grains by fungi. Bot. Rev. 23: 108–134

CICCARONE, A. (1960): Reproduction is affected – In: Plant Pathology, an advanced treatise, vol. 1., ed. J. G. Horsfall and A. E. Dimond. Academic Press, New York

COLHOUN, J., G. S. TAYLOR & C. S. MILLAR (1963): Disease of cereals caused by *Fusarium nivale*. Nature, Lond. 200: 597

COLHOUN, J. & D. PARK (1964): *Fusarium* diseases of cereals. I. Infection of wheat plants, with particular reference to soil moisture and temperature on seedling in-infection. Trans. Br. mycol. Soc. 47: 559–572

COLHOUN, J. (1964): Presidential address – Environment and plant disease. Trans. Br. mycol. Soc. 47: 1–13

Crosier, W. F. & S. W. Braverman (1963): Storage of fungi in seeds of small grains. Phytopathology **53**: 348

Csuti, E., J. M. Lemaire, J. Ponchet & F. Rapilly (1965): Exemples d'interaction fongiques au niveau de plantules de blé contaminées par l' *Helminthosporium sativum*. Annls. Épiphyt. 16: 37–44.

Dekker, J. (1957): Inwendige ontsmetting van door *Ascochyta pisi* aangetaste erwtezaden met de antibiotica rimocidine en pimaricine, benevens enkele aspecten van het parasitisme van deze schimmel. Tijdschr. Pl.Ziekt. **63**: 65–144

Denis, S. J., Charlotte J. Snodgrass, J. A. Koburger and E. S. Elliott (1966): An azide-rose bengal medium for the isolation of *Fusarium* species from red clover roots. Phytopathology **56**: 584

Doling, D. A. (1965): Some ecological factors influencing the proportion of loose smut (*Ustilago nuda* (Jens.) Rostr.) in wheat crops. Ann. appl. Biol. **33**: 303–306

Doyer, L. C. (1938): Manual for the determination of seed-borne diseases. Int. Seed Test. Assoc., Wageningen (59 pp.)

Düggeli, M. (1904): Die Bakterienflora gesunder Samen und daraus gezogener Keimpflänzchen. Zentbl. Bakt. ParasitKde Abt. 2, **12**: 602–614, 695–712; **13**: 56–63, 198–207

Endo, R. M. (1963): Influence of temperature on rate of growth of five fungus pathogens of turfgrass and on rate of disease spread. Phytopathology **53**: 857–861

Faber, F. C. von (1912): Das erbliche Zusammenleben von Bakterien und tropischen Pflanzen. Jahrb. Wiss. Bot. **51**: 285–375

Faber, F. C. von (1914): Die Bakteriensymbiose der Rubiaceen (Erwiderung und ergänzende Mitteilungen). Jahrb. Wiss. Bot. **54**: 243–265

Fenner, L. M. & L. R. Fate (1947): *Ceratostomella ulmi* on elm bark treated with 2,4-Dichlorophenoxyacetic-acid. Phytopathology **37**: 925–928

Fiala, F. (1963): Feldversuche mit schneeschimmelbefallenem Roggensaatgut im pannonischen und baltischen Klimagebiet. Bodenkultur **15**: 60–95

Flentje, N. T. (1959): The physiology of penetration and infection. – In: Plant Pathology, problems and progress, 1908–1958, ed. C. S. Holten et al. Un. of Wisconsin Press

Fritz, T. (1966): Influence of temperature and parasitic fungi on germinating capacity of cereal seed. Proc. int. Seed Test. Assoc. **31**: 711–717

Garcia-Acha, I., J. A. Leal & J. R. Villanueva (1966): Effect of sodium propionate on sporulation of *Fusarium culmorum*. Can. J. Microbiol. **12**: 1290

Garrett, S. D. (1956): Biology of root-infecting fungi. Cambridge University Press (293 pp.)

Garrett, S. D. (1966): Spores as propagules of disease. – In: The fungus spore, ed. M. F. Madelin, Proc. 18th Symp. Colston Research Soc., Butterworths, London (345 pp)

Gäumann, E. (1951): Pflanzliche Infektionslehre. Verl. Birkhaüser, Basel (681 pp.)

Gel'tser, F. Y. (1965): The significance of symbiotrophic microorganisms (endophytes) for the productivity of plants. In: Plant microbes relationships, ed. J. Macura & V. Vancura, Proc. Symp. Relationships between Soil Microorganisms and Plant Roots at Prague. Publ. House Czechoslovak Acad. Sci. (333 pp.)

Germ. H. (1960): Das Problem der Keimfähigkeitsbestimmung von Fusariumkran-

kem Weizen- und Roggensaatgut. Saatgutwirtsch. **12**: 164–165

GIBSON, I. A. (1957): Saprophytic fungi as destroyers of germinating pine seed. E. Afr. Agr. J. **22**: 203–206

GRÜNNEWIG, J. (1933): Beiträge zur Kenntnis des Loliumpilzes. Beitr. Biol. Pfl. **20**: 227–254

GUISCAFRÉ-ARRILLAGA, J. (1949): Inhibitory action of 2,4-Dichlorophenoxyaceticacid on *Penicillium digitatum* and *Phomopsis citri*. Phytopathology **39**: 8

HAGBORG, W. A. F., G. H. WARNER & N. A. PHILIPS (1950): Use of 2,4-D as an inhibitor of germination in routine examination of beans for seed-borne infection Science, N.Y. **111**:91

HARRIS, M. R. & C. W. ELLETT (1945): A *Penicillium* disease of soybeans. Phytopathology **35**: 144–145

HEALD, F. D. (1921): The relation of spore load to the percent of stinking smut appearing in the crop. Phytopathology **11**: 269–278

HEALD, F. D. & L. W. BOYLE (1923): Further notes on the relation of the spore load to the percentage of stinking smut appearing in the crop. Phytopathology **13**: 334–337

HEWETT, P. (1964): Testing carrot seed infected with *Alternaria porri* f. sp. *dauci*. Proc. int. Seed Test. Assoc. **29**: 463–471

HEWETT, P. (1965): A survey of seed-borne fungi of wheat. I. The incidence of *Leptosphaeria nodorum* and *Griphosphaeria nivalis*. Trans. Br. mycol. Soc. **48**: 59–72

HEWETT, P. (1967): A survey of seed-borne fungi of wheat. II. The incidence of common species of *Fusarium*. Trans. Br. mycol. Soc. **50**: 175–182

HOTTES, C. F. & W. A. HUELSON (1927): The determination of quality in sweet corn seed by means of the optical measurement of leached materials. J. agric. Res. **35**: 147–166

HSIA, Y. T. & J. J. CHRISTENSEN (1951): Effect of 2,4-D on seedling blight of wheat caused by *Helminthosporium sativum*. Phytopathology **41**: 1011–1020

HUBER, D. M. & R. D. WATSON (1966): How valid is the agar plate inhibition test for determining antagonism between soil microorganisms? Phytopathology **56**: 882

IKENBERRY, R. W. (1961): The isolation of *Fusarium moniliforme* Sheld. from corn kernels. Proc. Iowa Acad. Sci. **68**: 100–102

ISENBECK, K. (1939): Die Bedeutung der Faktoren Temperatur und Licht für die Frage der Resistenzverschiebung bei verschiedenen Sommergersten gegenüber *Helminthosporium gramineum*. Kühn Arch. **44**: 1–54

IVANOFF, S. S. (1958): The water-soak method of plant disease control in relation to microbial activities, oxygen supply, and food availability. Phytopathology **48**: 502–508

JONG, C. B. DE (1967): Identiteit, pathogeniteit en waardplantenreeks van een op zaad en kiemplanten van veldsla voorkomende *Phoma*-soort Unpubl. Rep. Agr. Un. Wageningen

JONGH, P. DE (1938): On the symbiosis of *Ardisia* crispa (Thumb.) ADC. Verh. Koninkl. Akad. Wetenschap, Afd. Natuurk., Sect. II, 37 No. 6

KIETREIBER, M. (1962): Der *Septoria*-Befall von Weizenkörnern (zur Methodik der

Erkennung). Proc. int. Seed Test. Assoc. **27**: 843–855

KIETREIBER, M. (1966a): Atypische *Septoria nodorum*-Symptome an Weizenkeimlingen (Das Verhalten der Sorte Probus). Proc. int. Seed Test. Assoc. **31**: 179–186

KIETREIBER, M. (1966b): Keimpotenz und Feldaufgang von kranker und behandeltem Getreidesaatgut. Bodenkultuur **17**: 50–55

KRASILNIKOV, N. A. & E. A. RAZNITZYNA (1946): A bacterial method of controlling damping-off of Scotch pine seedlings caused by *Fusarium*. Agrobiologiya **5–6**: 109–121

KREITLOW, K. W., C. L. LEFEBVRE, J. T. PRESLEY & W. J. ZAUMEYER (1961): Diseases that seeds can spread. – In: Seeds, U.S.D.A. Yearbook 1961, Washington, D.C.

KULIK, M. M. & M. F. COMBS (1967): Carbon and nitrogen nutrition of some seed-borne fungi in vitro. Proc. int. Seed Test. Assoc. 32: 591–602.

LANGE, R. T. (1966): Bacterial symbiosis with plants. – In: Symbiosis, vol. I. Associations of Microorganisms, Plants, and Marine Organisms, ed. S. Mark Henry. Academic Press, New York

LEACH, C. M. (1959): Effects of visible and ultraviolet radiations on the sporulation of *Ascochyta pisi* and other seed-borne fungi. Phytopathology **49**: 543

LEACH, C. M. (1962): Sporulation of diverse species of fungi under near-ultraviolet radiation. Can. J. Bot. **40**: 151–161

LEACH, C. M. (1967a): Interaction of near-ultraviolet light and temperature on sporulation of the fungi *Alternaria, Cercosporella, Fusarium, Helminthosporium* and *Stemphylium*. Can. J. Bot. **45**: 1999–2016

LEACH, C. M. (1967b): The light factor in the detection and identification of seed-borne fungi. Proc. int. Seed Test. Assoc. **32**: 565–589

LEBEN, C. (1965): Epiphytic microorganisms in relation to plant disease. A. Rev. Phytopath. **3**: 209–230

LEDINGHAM, R. J., B. J. SALLANS & P. M. SIMMONDS (1949): The significance of the bacterial flora of wheat seed in inoculation studies with *Helminthosporium sativum*. Scient. Agric. **29**: 253–262

LIMONARD, T. (1966a): De factor 'substraatvochtigheid' in het gezondheidsonderzoek van zaaizaden. Meded. Rijksproefstn. Zaadcontrole No. **17**: 59–63

LIMONARD, T. (1966b): A modified blotter test for seed health. Neth. J. Pl. Path. **72**: 319–321

LIMONARD, T. (1967a): Bacterial antagonism in seed health tests. Neth. J. Pl. Path. **73**: 1–14

LIMONARD, T. (1967b): Some remarks on the preparation of agar tests in seed health investigation. Proc. int. Seed Test. Assoc. **32**: 603–614

LIMONARD, T. (1968): Verbesserung der Flieszpapiermethode für die Gesundheitsprüfung von Samen. Umschau in Wissensch. u. Technik **68**: 122–123

LITTMANN, M. L. (1947): A culture medium for the primary isolation of fungi. Science, N. Y. **106**: 109–111

MAAS GEESTERANUS, H. P. (1960): *Pyrenophora calvescens* op blauwmaanzaad. Tijdschr. Pl.Ziekt. **66**: 237–248

MACHACEK, J. E. & H. A. H. WALLACE (1942): Non-sterile soil as a medium for

tests of seed germination and seed-borne disease in cereals. Can. J. Res., Sect. C., **20**: 539–557
MACHACEK, J. E., W. J. CHEREWICK, H. W. MEAD & W. C. BROADFOOT (1951): A study of some seed-borne diseases of cereals in Canada. II. Kind of fungi and prevalence of disease in cereal seed. Scient. Agric. **31**: 193–206
MACHACEK, J. E., H. A. H. WALLACE & W. C. BROADFOOT (1954): A study of some seed-borne diseases of cereals. III. Effect of rate of seeding, percentage of infected kernels, and weeds, on the yield of plots with treated and untreated seeds. Can. J. agric. Sci. **35**: 240–251
MALONE, J. P. (1962): Studies on seed health. IV. The application of heat to seed oats as an aid in the detection of *Pyrenophora avenae* by the Ulster method. Proc. int. Seed Test. Assoc. **27**: 856–861
MALONE, J. P. & A. E. MUSKETT (1964): Seed-borne fungi. Description of 77 fungus species. Proc. int. Seed Test. Assoc. **29**: 177–384
MALONE, J. P. & A. E. MUSKETT (1967): A review of the development of seed pathology. Pans **13**: 266–275
MALONE, J. P. (1968): Mercury-resistant *Pyrenophora avenae* in Northern Ireland seed oats. Pl. Path. **17**: 41–45
MARTIN, J. P. (1950): Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci. **69**: 215–232
MATTHEWS, S. & R. WHITBREAD (1968): Factors influencing pre-emergence mortality in peas. I. An association between seed exudates and the incidence of pre-emergence mortality in wrinkle-seeded peas. Pl. Path. **17**: 11–17
MEAD, H. W., W. C. BROADFOOT & J. E. MACHACEK (1950): A study of some seed-borne diseases of cereals in Canada. I. Comparison of seed testing methods. Scient. Agric. **30**: 350–360
MEAD, H. W. & M. W. CORMACK (1961): Studies on *Ascochyta imperfecta* Peck. Parasitic strains among fifty isolates from Canadian alfalfa seed. Can. J. Bot. **39**: 793–797
MCKAY, R. (1952): Failure in oats due to root rot caused by *Gibberella zeae*. Scient. Proc. R. Dubl. Soc. **26**: (N.S.): 55–62
MCLENNAN, E. (1920): The endotrophic fungus of *Lolium* I. Proc. R. Soc. Vict. **32** (N.S.) 2: 252–301
MIEHE, H. (1914): Weitere Untersuchungen über die Bakteriensymbiose bei *Ardisia crispa*. I. Die Mikroorganismen. Jahrb. Wiss. Bot. **53**: 1–54
MIEHE, H. (1919): Weitere Untersuchungen über die Bakteriensymbiose bei *Ardisia crispa*. II. Die Pflanze ohne Bakterien. Jahrb. Wiss. Bot. **58**: 29–65
MILLER, J. J., D. J. PEERS & R. W. NEAL (1951): A comparison of the effects of several concentrations of oxgall in platings of soil fungi. Can. J. Bot. **29**: 26–31
MORTON, D. J., E. TOOL & I. K. HAGEN (1960): Procedure and effectiveness of the barley embryo test for loose smut used in North Dakota. Pl. Dis. Reptr. **44**: 802–803
NASH, S. M. & W. C. SNYDER (1962): Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology **52**: 567–572
NEERGAARD, P. Årsberetn. Frøpat. Kontr. 1953–1954, København, (17 pp.)
NEERGAARD, P. (1961): Uniformity in seed health testing. Rep. 6th. Commonwealth

Mycological Conference, Kew: 61–67; Commonw. Mycol. Inst., Kew

NEERGAARD, P. (1962): Tolerances in seed health testing – a discussion of basic principles. Proc. int. Seed Test. Assoc. **27**: 386–399.

NEERGAARD, P. & A. SAAD (1962): Seed health testing of rice. A contribution to development of laboratory routine testing methods. Indian Phytopath. **15**: 85–111

NEERGAARD, P. (1965): Historical development and current practices in seed health testing. Proc. int. Seed Test. Assoc. **30**: 99–118

NEILL, J. C. (1941): The endophytes of *Lolium* and *Festuca*. N. Z. Jl. Sci. Technol. A23: 185–193

NEWHOOK, F. J. (1951): Microbiological control of *Botrytis cinerea* Pers. I. The role of pH changes and bacterial antagonism. Ann. appl. Biol. **38**: 169–184 II. Antagonism by fungi and actinomycetes. Ann. appl. Biol. **38**: 185–202

NOBLE, Mary, J. DE TEMPE & P. NEERGAARD (1958): An annotated list of seed-borne diseases. Commonw. Mycol. Inst., Kew, (159 pp.), Proc. int. Seed Test. Assoc. **24**: 1–159

NOBLE, M. (1965): Communication at 8th. workshop of ISTA-P.D.C. at Belfast

NOBLE, M., Q. D. MACGARVIE, A. F. HAMS & E. L. LEAFE. (1966): Resistance to mercury of *Pyrenophora avenae* in Scottish seed oats. Pl. Path. **15**: 23–28

NOVOGRUDSKII, D., E. BERESOVA, M. NAKHIMOVSKAYA & M. PERVIAKOVA (1937): The influence of bacterization of flax-seed on the susceptibility of seedlings to infection with parasitic fungi. Dokl. Akad. Nauk. S.S.S.R. (= C.r. Acad. Sci. U.R.-S.S.) **14**: 385–388 (Rev. appl. Mycol. **16**: 676)

PAPAVIZAS, G. C. (1967): Evaluation of various media and antimicrobial agents for isolation of *Fusarium* from soil. *Phytopathology* **57**: 848–852

PEPPER, E. H. & R. L. KIESLING (1963): A list of bacteria, fungi, yeasts, nematodes and viruses occurring on and within barley kernels. Proc. Assoc. off. Seed Analysts N. Am. **53**: 199–207

PONCHET, J. (1960): La détection des parasites transmis par les semences de blé. Proc. int. Seed Test. Assoc. **25**: 539–553

PONCHET, J. (1966): Étude des communautés mycopéricarpiques du caryopse de blé. Annls. Epiphyt. 17 (hors série 1) (112 pp.)

POPP, W. (1959): A new approach to the embryo test for predicting loose smut of wheat in adult plants. Phytopathology **49**: 75–77

PORTER, C. L. (1924): Concerning the character of certain fungi as exhibited by their growth in the presence of other fungi. Am. J. Bot. **11**: 168–188

POUND, G. S. (1947): Variability in *Phoma lingam*. J. agric. Res. **75**: 113–133

PRESLEY, J. T. (1958): Relation of protoplast permeability to cottonseed viability and predisposition to seedling diseases Pl. Dis. Reptr. **42**: 852

RUSSELL, R. C. (1950): The whole-embryo method for testing barley for loose smut as a routine test. Scient. Agric. **30**: 361–366

RUSSELL, R. C. & W. POPP (1951): The embryo test as a method of forecasting loose smut infection in barley. Scient. Agric. **31**: 559–565

SAMPSON, K. (1935): The presence and absence of an endophytic fungus in *Lolium temulentum* and *L. perenne*. Trans. Br. mycol. Soc. **19**: 337–343

SCHERMER, A. K. F. & B. SCHIPPERS (1965): Overdracht van *Verticillium albo-atrum* met zaad van *Senecio vulgaris*. Neth. J. Pl. Path. **71**: 62

SCHIPPERS, B. & A. K. F. SCHERMER (1966): Effect of antifungal properties of soi on dissemination of the pathogen and seedling infection originating from *Verticillium*-infected achenes of *Senecio*. Phytopathology **56**: 549–552

SCHROTH, M. N. & R. J. COOK (1964): Seed exudation and its influence on pre-emergence damping-off of bean. Phytopathology **54**: 670–673

SIMMONDS, J. H. (1963): Studies in the latent phase of *Colletotrichum* species causing ripe rots of tropical fruits. Qd. J. agric. Sci. 20: 373–424

SIMMONDS, P. M. (1947): The influence of antibiosis in the pathogenicity of *Helminthosporium sativum*. Scient. Agric. **27**: 625–632

SLYKHUIS, J. T. (1947): Studies on *Fusarium culmorum* blight of crested wheat and bromegrass seedlings. Can. J. Res., Sect. C. **25**: 155–180

SMITH, N. R. & V. T. DAWSON (1944): The bacteriostatic action of rose bengal in media used for plate counts of soil fungi. Soil Sci. **58**: 467–471

SPEK, J. VAN DER (1965): *Botrytis cinerea* als parasiet van vlas. Doctor's Diss. Agric. Univ. Wageningen (146 pp.)

STEINER, G. W. & R. D. WATSON (1965): Use of surfactants in the soil dilution and plate count method. Phytopathology **55**: 728–730

TAKAYANAGI, K. & K. MURAKAMI (1968): New method of seed viability test with exudates from seed. Preprint No. 25, 15th. Int. Seed Test. Congress, 1968, New Zealand.

TATUM, L. A. (1954): Seed permeability and cold-test reaction in *Zea mays*. Agron. J. **46**: 8–10

TEMPE, J. DE (1958a): *Botrytis* infection of flax seed. Proc. int. Seed Test. Assoc. **23**: 316–331

TEMPE, J. DE (1958b): Infectie van lijnzaad met *Botrytis* – waardering en bestrijding. Tijdschr. PlZiekt. **64**: 281–296

TEMPE, J. DE (1958c): Three years of field experiments on seed-borne diseases and seed treatment of cereals. Proc. int. Seed Test. Assoc. **23**: 38–67

TEMPE, J. DE (1961a): De *Phoma lingam*-infectie van koolzaden. Meded. Rijksproefstn. Zaadcontrole No. **13**: 116–118

TEMPE, J. DE (1961b): Beoordeling van lijnzaad op *Botrytis*. Tijdschr. Pl.Ziekt. **67**: 554–561

TEMPE, J. DE (1963a): Health testing of flax seed. Proc. int. Seed Test. Assoc. **28**: 107–131

TEMPE, J. DE (1963b): On methods of seed health testing – principles and practice. Proc. int. Seed Test. Assoc. **28**: 97–105

TEMPE, J. DE (1963c): The use of correlation coefficients in comparing methods for pathogenic elements. Proc. int. Seed Test. Assoc. **28**: 153–165

TEMPE, J. DE (1964): Seed-borne *Fusarium* infection in temperate climate cereals. Proc. int. Seed Test. Assoc. **29**: 97–116

TEMPE, J. DE & T. LIMONARD (1966): The influence of substrate moisture on the results of seed health testing in blotter medium. Proc. int. Seed Test. Assoc. **31**: 169–178

TIFFANY, L. H. (1951): Delayed sporulation of *Colletotrichum* on soybean. Phytopa-

thology **41**: 975–985

TVEIT, M. & M. B. MOORE (1954): Isolates of *Chaetomium* that protect oats from *Helminthosporium victoriae*. Phytopathology **44**: 686–689

TVEIT, M. & R. K. S. WOOD (1955): The control of *Fusarium* blight in oat seedlings with antagonistic species of *Chaetomium*. Ann. appl. Biol. **43**: 538–552

TYNER, L. E. (1953): The control of loose smut of barley and wheat by spergon and by soaking in water at room temperature Phytopathology **43**: 313–316

TYNER, L. E. (1957): Factors influencing the elimination of loose smut from barley by water-soak treatments. Phytopathology **47**: 420–422

VERONA, O. (1963): Interaction entre la graine en germination et les microorganismes telluriques. Annls. Inst. Pasteur, Paris. **105**: 75–98

VÖRÖS, J., Z. KIRÁLY & G. L. FARKAS (1957): Role of polyphenolase in streptomycin-induced resistance to *Phytophthora* in potato. Science, N.Y. **126**: 1178

WAGGONER, P. E. (1956): Chemical treatment of potato seed in Connecticut 1955. Pl. Dis. Reptr. **40**: 411–413

WALLACE, R. H. & A. G. LOCHHEAD (1951): Bacteria associated with seeds of various crop plants. Soil Sci. **71**: 159–166

WALLEN, V. R. (1964): Host-parasite relations and environmental influences in seed-borne diseases. In: Microbial behaviour, 'in vivo' and 'in vitro'.. ed. H. Smith & J. Taylor, Cambridge Un. Press, London (296 pp.)

WIESNER, K. (1965): Der Besatz von *Beta*-Rübensaatgut mit Wurzelbranderregern und der Einflusz von Umweltfaktoren auf das Auftreten des samenbürtigen Wurzelbrandes. Arch. PflSchutz. **1**: 131–154

PLATE I

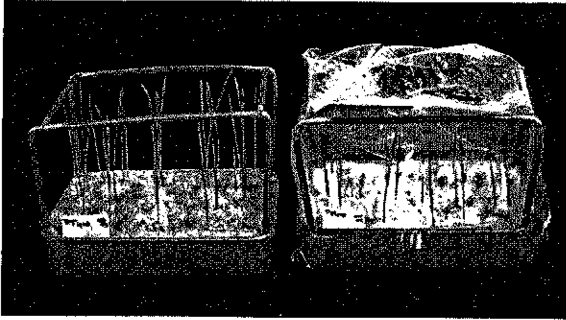


Fig. 1. 'Tents' for sand and soil tests (cf. section 2.2.3)

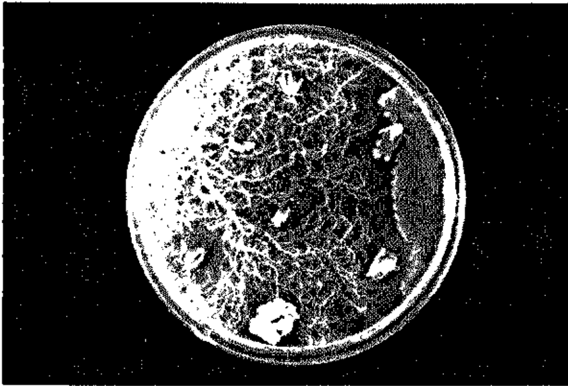
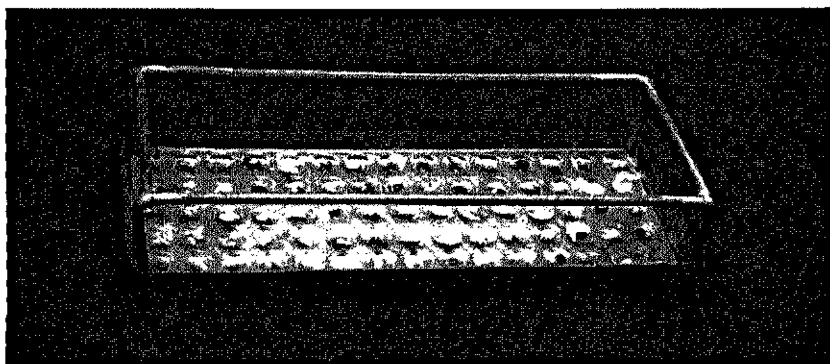


Fig. 2. *Bacillus cereus* and *Bacillus cereus* var *mycoides* growing from wheat seeds on nutrient agar (cf. section 3.16)

PLATE II



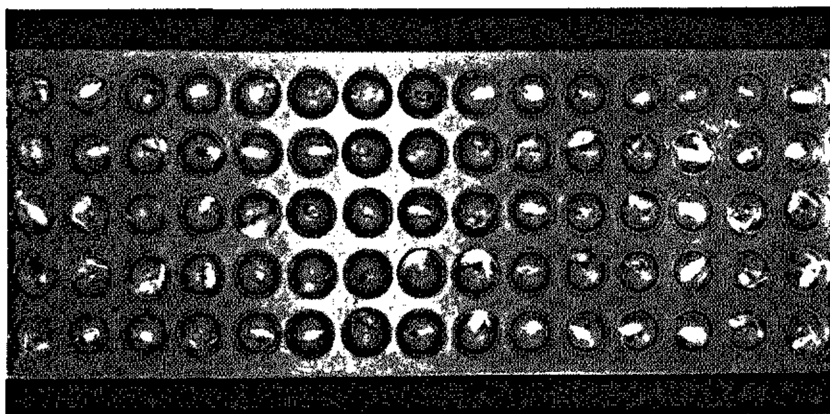


Fig. 1, 2 and 3. Materials for perspex-freezing method with wheat seed (cf. section 4.12)

PLATE III

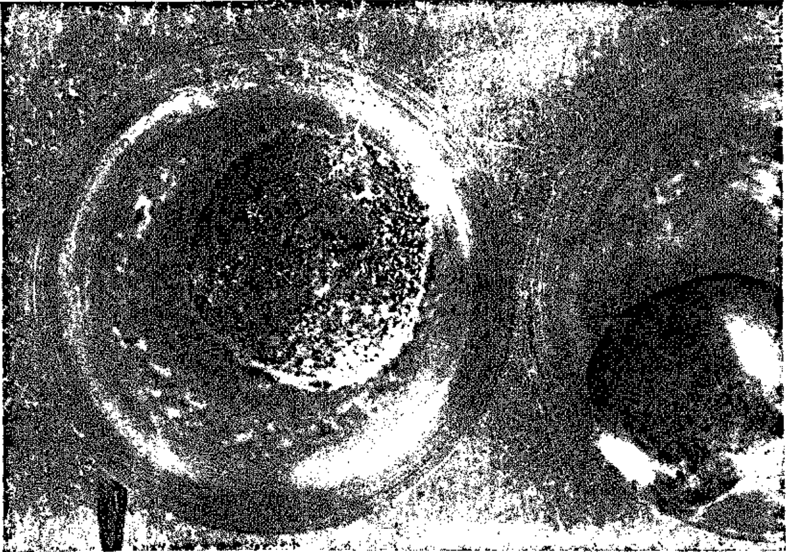


Fig. 1. Detail of frozen pea-seed with *Ascochyta pisi* tested by means of perspex-freezing method (cf. section 4.12)

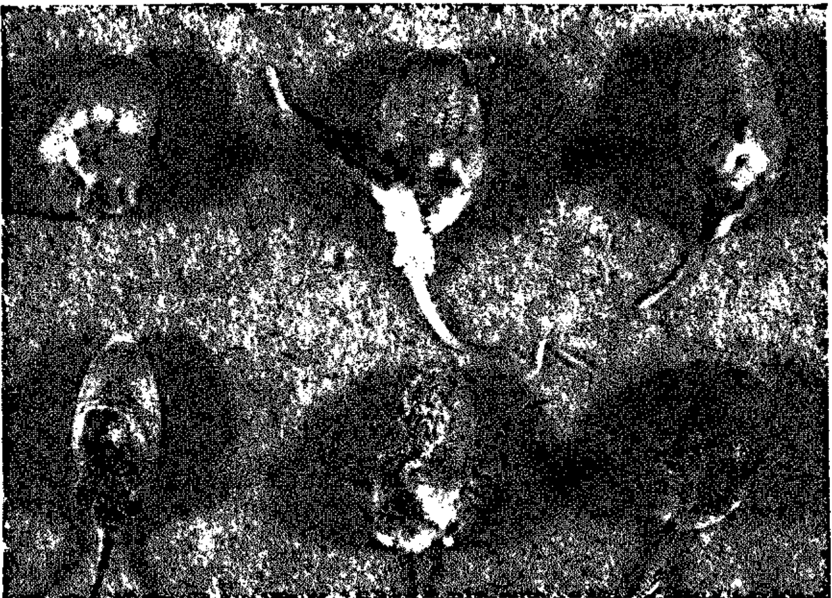


Fig. 2. Frozen wheat seeds with *Septoria nodorum* (cf. section 4.9.2)

PLATE IV

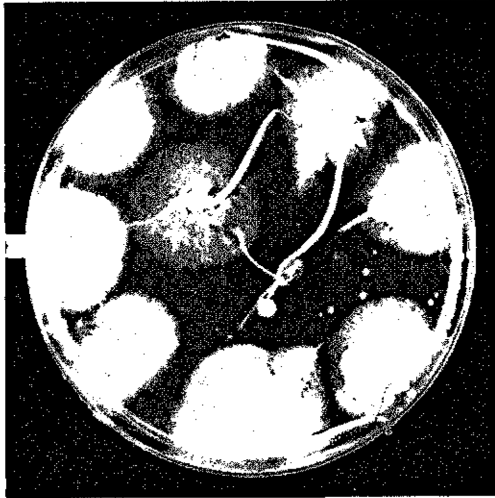


Fig. 1. Eight colonies of *Fusarium graminearum* and one of *F. poae* growing from wheat seeds on peptone-PCNB agar (cf. section 5.6)

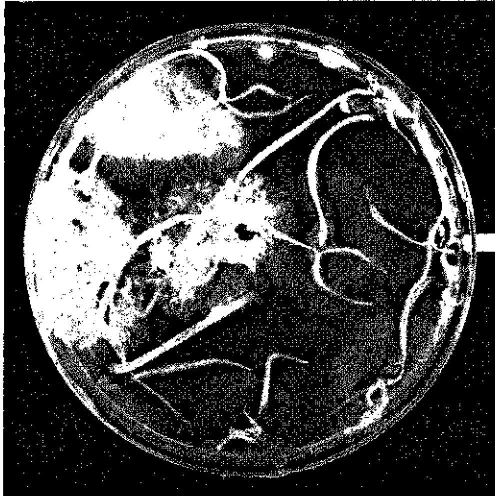
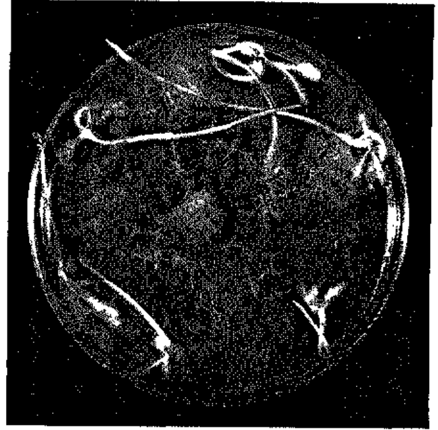


Fig. 2. Three colonies of *Fusarium graminearum* and four of *F. nivale* growing from wheat seeds on peptone-PCNB agar (cf. section 5.7)

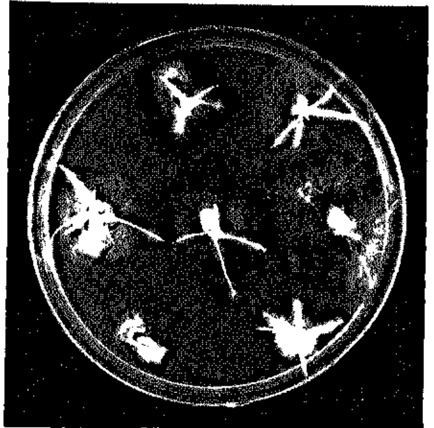
PLATE V

a.



a.

b.



b.

Fig. 1. Two examples of the effect of the addition of oxgall to PDA on the interference of other fungi with the manifestation of *Septoria nodorum* growing from wheat seeds (cf. section 5.7)
a. PDA without oxgall; b. PDA + 2000 p.p.m. oxgall