MYCOSPHAERELLA GRAMINICOLA ON WHEAT

GENETIC VARIATION AND HISTOPATHOLOGY

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Mycosphaerella graminicola on Wheat

Genetic variation and Histopathology

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 25 juni 1996
des namiddags te dertien uur dertig in de Aula
van de Landbouwuniversiteit te Wageningen

IPO-DLO

STELLINGEN

bij het proefschrift

"MYCOSPHAERELLA GRAMINICOLA ON WHEAT -
GENETIC VARIATION AND HISTOPATHOLOGY"

1 De gen-om-gen hypothese vertoont een treffende overeenkomst met het principe van oog-om-oog en tand-om-tand.

2 Gezien de vrijwel continu optredende genetische recombinatie in het pathogeen is het niet zo zeer de vraag of het *Mycosphaerella graminicola* - tarwe pathosysteem beantwoordt aan de gen-om-gen hypothese, maar veeleer wat het epidemiologisch belang van deze hypothese en de daaraan verbonden interacties is.
Dit proefschrift

3 De veronderstelling dat resistentie van tarwe tegen *Mycosphaerella graminicola* berust op remming van de pycnidiumproductie is onjuist.
Dit proefschrift
Cohen, L., and Eyal, Z. 1993. Plant Pathology 42:737-743

4 De adaptatie van *Mycosphaerella graminicola* isolaten aan enerzijds broodtarwe en anderzijds aan durumtarwe is mogelijk gerelateerd met op het mitochondriaal DNA aanwezige kenmerken, hetgeen onderzocht dient te worden in kruisingsexperimenten tussen deze varianten.
Dit proefschrift

5 De kwalitatieve aspecten in het door velen als kwantitatief beschouwde tarwe-*Mycosphaerella graminicola* pathosysteem zouden wel eens veel groter kunnen zijn dan wordt verondersteld.
Dit proefschrift

6 De rol van de ascosporen in de epidemiologie van *Mycosphaerella graminicola* is onderschat.
Dit proefschrift
Shaw, M.W., and Royle, D.J. 1993. Plant Pathology 42:882-899. Royle, D.J. 1994. Plant Pathology 43:777-789.

7 Wetenschap vertoont de kenmerken van geloof

8 De apostel Paulus omschreef in Hebreeën 11 *het* geloof als 'een vaste grond der dingen die men hoopt en een bewijs der zaken die men niet ziet'. Elke wetenschapper zou hier, in verband met de vorige stelling, goede notie van moeten nemen.

9 Het KEMA-keurmerk dient alleen te worden gebruikt ter kwalificatie van electrische materialen.
Met andere ogen, over wetenschap en het zoeken naar zin. A. van den Beukel, 1994, Ten Have, Baarn.

10 Wetenschap is veeleer een vlucht dan dat zij een vlucht neemt.

11   Transgressie voor resistentie tegen gele roest in tarwe kan ook worden verklaard door uitsplitsende onderdrukkergenen.
Kema, G.H.J., Lange, W., and Van Silfhout, C.H. 1995. Phytopathology 85:425-429.

12   De mogelijkheid om persoonsnamen toe te kennen aan land-, tuinbouw en bloemisterijgewassen dient, in verband met het waarborgen van de integriteit van deze personen, heroverwogen te worden.
Roebroeck, E.J.A., and Mes, J.J. 1992. Neth. J. Pl. Path. 98:57-64.

13   Het rangschikken van *Septoria tritici* onder de zgn. 'soilborne pathogens' is een feitelijke onjuistheid en doet bovendien geen recht aan het belang van de 'airborne' ascosporen in het tarwe-*Mycosphaerella graminicola* pathosysteem.
Parlevliet, J.E., 1993. Durability of disease resistance. Kluwer Academic Publishers, Dordrecht.

14   De werking van het 'marktmechanisme' is asociaal.

15   De 'wegwerp cultuur' strekt zich uit tot het menselijk leven.

16   Het Nederlands asielbeleid wordt gekenmerkt door 'vriendjespolitiek'.

17   Het sociaal gedrag van Afrikaanse leeuwen en mensen vertoont een treffende overeenkomst: leiders en angsthazen.
Heinsohn R.,H., and Packer, C. 1995. Science 269:1260-1262

GERRIT HAATJE JAN KEMA
WAGENINGEN, 25 JUNI 1996

Aan mijn ouders,

voor Hanna,
Dorine, Jan-Harm, Joanne, Maaike, Hilde en Loes

De vreze des HEEREN is het beginsel der wetenschap (Spreuken 1:7)

ABSTRACT

The research described in this thesis was focused on a comprehensive understanding of the generation and extent of genetic variation, and its effects on host cultivars in the wheat-*Mycosphaerella graminicola* pathosystem. Inoculation experiments were conducted in the seedling stage and adult plant stages under field conditions over several years. These experiments encompassed nearly 100 isolates of the pathogen tested on some 50 wheat cultivars. Parametric and non-parametric statistical analyses were employed on large data and very small data sets, and indicated that in all experiments cultivar x isolate interactions were significant. Pathogen isolates originating from bread wheat and durum wheat appeared to be adapted to their hosts, respectively. Molecular analysis of the interternally transcribed spacer regions of isolates from both forms, indicated that these were taxonomically closely related. The inoculation experiments indicated that specificity is an important characteristic of the pathosystem. After having determined the wide genetic variation in the pathosystem, experiments were conducted to elucidate the mating system of the pathogen. The results indicated a bipolar heterothallic mating system and RAPD analyses of progenies showed regular Mendelian inheritance. Furthermore it was shown that the pathogen is able to complete several generative cycles within a season. Hence, genetic recombination appears to be the driving force behind the determined vast genetic variation. Biochemical and histological experiments were conducted involving compatible and incompatible interactions with the host plant in order to elucidate the resistance mechanism of wheat to the pathogen.

Additional key words : genetics, *Septoria tritici, Triticum* ssp.

PREFACE

Het in dit proefschrift beschreven onderzoek is het resultaat van intensieve samenwerking. De basis werd gelegd in een samenwerkings-projekt over septoria tritici bladvlekkenziekte waarin het IPO-DLO, de Universiteit van Tel Aviv en het CIMMYT in Mexico participeerden. Inspirators van dat projekt waren Zahir Eyal, Maarten van Ginkel en Cor van Silfhout. Jullie enthousiasme heeft bij mij een interesse gewekt die zich in de loop van het onderzoek alleen maar verdiept heeft. Hiervoor ben ik jullie bijzonder dankbaar. De vele discussies over de eigenaardigheden van het tarwe-*Mycosphaerella graminicola* pathosysteem die we vooral voerden tijdens de door ons georganiseerde workshops waren voor mij een stimulans om het onderzoek uit te voeren op de in dit proefschrift beschreven manier.

Zahir, your enthusiasm for research on *Mycosphaerella graminicola* initiated an important project, and I'm grateful for the opportunities it provided. Our tough discussions never affected your warmth and friendship. Thanks for that. Maarten, de door jou gecoördineerde workshop in Ethiopië heeft mij geleerd welk effect een pathogeen kan hebben op het dagelijks leven van de kleine boer. Jouw bevlogenheid heeft in grote mate bijgedragen tot het succes van dit onderzoek. Vooral omdat we het niet eens waren. Je regelmatige visites (of waren het visitaties ?) vormden een wederzijds genoegen, al liet ik je weleens wachten.

Cor, we kennen elkaar al jaren. Jij hebt mijn wetenschappelijke ontwikkeling voor een groot gedeelte mogelijk gemaakt. In de eerste plaats heb je, reeds tijdens een stage in 1980, samen met Ron Stubbs, mijn interesse gewekt voor gele roest. Die belangstelling is nooit meer over gegaan en heeft zich door de jaren alleen maar verdiept. Ik kon mede door de vrijheid die je mij gaf, naast mijn werk aan de LUW gaan studeren. Tijdens die periode veranderde er voor ons beide veel. Zo werd ik van assistent onderzoeker, een toen niet alledaagse gang van zaken. Dat was alleen mogelijk dankzij het vertrouwen en de vrijheid die je mij in die periode hebt gegeven om het onderzoek op mijn wijze in te vullen en geleidelijk over te nemen. Ik ben je hiervoor nog steeds erg dankbaar, zonder de stimulerende rol van Orlando de Ponti daarbij te vergeten.

I already mentioned that the research described in this thesis was a cooperative enterprise. Peter Arama, Juan Annone, Rachid Sayoud, DaZhao Yu and Maria Todorova participated in the experiments over a period of five years. Peter, you chose the host, I the pathogen. I wish you every success. Juan, mi buen amigo de las pampas argentinas, the many hours we spent in the old greenhouse observing thousands of plants are the foundation of this thesis. Many questions were raised and we were often enjoying the different responses of the cultivars we studied. The necessary breaks were usually accompanied by 'tompouches', and they were usually followed by profound discussions about the reason why ? I'm glad we have met each other several times afterwards and I'm looking forward to the next meeting. Rachid, bon ami, your blend of Algeria and

France created a pleasant atmosphere in our room for more than a year. Your sense of the real field work emphasized the differentiation of bread wheat and durum wheat adapted *Mycosphaerella graminicola* strains. It's great that your period with us contributed to your PhD in Winnipeg. DaZhao, dear friend, I will never forget your devotion when trying to find answers to our pathological questions by histological studies. We will always remind the chinese dishes you prepared at our home, and the fortnightly Friday evening Bible studies, which opened another world for you. It's nice that your PhD programme in Wageningen will enable regular visits to The Netherlands. Dear Maria, you joined us for a relatively short, but extremely interesting period just after we succeeded in making crosses between *Mycosphaerella graminicola* isolates. I enjoyed your enthusiasm for *Mycosphaerella graminicola*, which is rare among rust adepts.

Beste Joop de Bree, jouw kennis van de statistiek heeft mij vaak behoed voor geheel nieuwe bijdragen aan dit vakgebied. Ik heb je bijdragen aan het onderzoek altijd erg gewaardeerd en denk nog vaak aan de discussies die we voerden. Pieter, jouw hulp bij de laatste statistische loodjes was onontbeerlijk. Beste Frits Rijkenberg, hartelijk bedankt voor je gastvrijheid, openheid en voor je interesse om *Mycosphaerella graminicola* histologie uit te breiden met SEM en TEM. We zijn er nog niet klaar mee en ik zie uit naar een verdere verdieping van dit onderzoek. Beste Els, hartelijk dank voor de systematische wijze waarop je de laatste jaren aan het onderzoek hebt bijgedragen. Ik bewonder je geduld met mijn optimisme. Alle collega's binnen en buiten de sectie, afdeling, concentratie-onderwerp, of hoe het nu moge heten, heel hartelijk bedankt voor de prettige sfeer die het werk positief heeft beïnvloed. Beste Robert, de vele gesprekken die we samen hebben gevoerd gingen, gelukkig, niet altijd alleen over planteziekten. Ze waren voor mij steeds een verademing. Hans, ouwe nematoloog, bedankt voor je interesse. Het was altijd stimulerend om van gedachten te wisselen. Beste Cees, jouw bijdrage aan het onderzoek heb ik erg gewaardeerd. Het loopt prima, fijn dat we door kunnen gaan. Maarten en Lute-Harm, ik zie uit naar de verdere samenwerking. Lammert en Richard, bedankt voor de discussies die de resultaten van het onderzoek in een praktische context plaatsten.

Dear Bruce McDonald, James Brown and Mike Shaw, the contacts with all of you resulted in cooperative research efforts, which are invaluable for the perception of the character of *Mycosphaerella graminicola* on wheat.

Geachte Professor Zadoks, de manier waarop u mijn manuscripten beoordeelde was altijd een stimulans om snel door te gaan. Hartelijk dank voor de vormende gesprekken die we tijdens die sessies voerden.

Bij de uitvoering van de experimenten was de onvermoeibare hulp van de kassendienst, de technische dienst en de proefvelddienst onmisbaar.

Familie en vrienden, jullie belangstelling heeft me meer gedaan dan jullie konden bevroeden. Steven, hartelijk dank voor het corrigeren van het hele manuscript. Pa en Ma, jullie waren de eerste mensen in mijn leven en hebben mijn opleiding mogelijk gemaakt. Heel, hartelijk dank

hiervoor, maar vooral voor het karakter van de warme huiselijke kring die een mens anders vormt, maar met een veel diepere uitwerking.

Mijn hele studie, en dus ook het tot stand komen van dit proefschrift was onmogelijk geweest zonder de jarenlange, voortdurend onbaatzuchtige ondersteuning van jou, lieve Hanna, en jullie lieve jongens. Het was niet altijd gemakkelijk, er is veel gebeurd, we hebben veel, heel veel gekregen. Jullie liefde was en is onontbeerlijk. Bovenal, dank ik de Heere God, die mij de kracht, de gezondheid en het inzicht gaf om dit werk te doen. *Soli Deo Gloria.*

Gert Kema
Wageningen, 8 Maart, 1996

CONTENTS

GENERAL INTRODUCTION

WHEAT

NOWADAYS WHEAT is culti-
vated throughout the
world and is largely re-
presented by two species,
bread wheat (*Triticum
aestivum* L.) and durum
wheat (*T. turgidum* (L.) Thell. spp.
durum L.). In the beginning of history,
wheat cultivation was confined to the
Middle-East where these species evol-
ved from wild progenitors and were
eventually domesticated (35). The wild
progenitors are the diploids *T. mono-
coccum* L. (AA, $2n=14$) and *Aegilops
squarrosa* L. (DD, $2n=14$), and the
tetraploid *T. turgidum* (L.) Thell. ssp.
dicoccoïdes (Körn.) Thell. (AABB,
$2n=28$). The evolution of cultivated
wheat and its distribution around the
world is an exciting, biological, agro-
nomical and cultural story (58,59).
Ancient empires were largely depen-
dent on wheat and barley cultivation,
and consolidated their power by tra-
ding. The importance of wheat was
already exemplified in the biblical
story of Jacob and his sons who relied
on the Egyptian wheat production.
Remnants of wheat crops from that era
have been regularly found in archaeo-
logical excavations (5,48,101,144).

INITIALLY, *T. MONOCOCCUM* and *T.
turgidum* ssp. *dicoccum*, a mutant from
T. turgidum ssp. *dicoccoïdes* with a non-
brittle rachis, were the major species,
of which the cultivation was concen-
trated in the Middle-East. Natural hy-
bridization between wild species may
lead to new species of higher ploidy
levels (35,58). Such a cross fertilization
between *T. turgidum* ssp. *dicoccum* and

Aegilops squarrosa, followed by poly-
ploidization resulted in the hexaploid
species *T. aestivum* (AABBDD, $2n=42$),
a process which is currently reprodu-
ced in laboratories and of considerable
practical and scientific interest (62).
The distribution area of *Aegilops
squarrosa* is much larger (Fig. 1) than
that of the other progenitors and
includes highly contrasting environ-
ments. Hence the donation of the D-
genome provided a vast adaptation
potential to bread wheat, enabling its
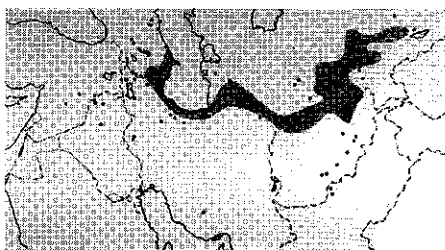
Fig. 1 Supposed ancient distribution area of *Ae.
squarrosa* (shaded area). Dots represent con-
temporary collection sites (after 144).

cultivation in most areas of the world.
Domestication was most probably
accompanied by unconcious selection
for non-brittleness of the rachis, which
enabled mechanical harvest. One of the
first hexaploid bread wheat species
formed according to the aforemen-
tioned process was *T. aestivum* ssp.
spelta, spelt wheat, a primitive species
that most probably originated in Iran
(59,70), and migrated to Europe where
it has been of considerable agricultural
importance until the beginning of the
twentieth century. Spelt wheat can still
be found in Switzerland, Belgium and
Luxemburg (Kema, personal obser-
vation) in the form of traditional
landraces that descend from ancient

Iranian ancestors. The distribution of this species was largely due to the Romans (47,59).

THE DOMESTICATED form of *T. turgidum* ssp. *dicoccum* is durum wheat. The cultivation of this species is of major importance in the Mediterranean area. It is particularly processed in North African traditional dishes as cous cous, and by the Italian food industry producing pastas. Both species of wheat are dealt with in this thesis.

THE GLOBAL importance of wheat for human consumption cannot be underestimated. Thirty-five per cent of the world's population consumes wheat as a staple food, which is higher than the percentages for maize and rice (each 20%). The global growth rate of wheat consumption is also higher than that of maize and rice, particularly in de developing countries. This demand will continue to increase in the future since population growth in developing countries is in an upward motion. It is estimated that within one century only 10% of the global population will be living in the so-called 'high-income countries' (91). Moreover, in contrast to other cereals such as rice and maize, wheat is considered as a convenience food. In addition to that, rising incomes also contribute to the increased global demand for wheat (15). This is also illustrated by the fact that despite the increasing share of developing countries in the total wheat production, their imports increase continually (Fig. 2). The total volume of wheat traded is approximately 21% of the total wheat production. More than 90% of this flow is contributed by
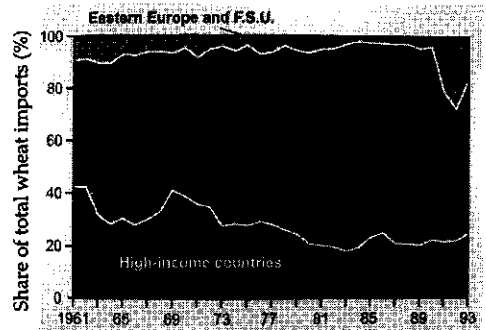
Fig. 2 World wheat imports, 1961-93 (CIMMYT, 1995).

the high-income countries.

POLITICAL CHANGES in the former Soviet Union recently had dramatic negative effects on wheat production, and thus on the distribution of the world's total wheat production. Hence, cereal imports to that region had serious repercussions on prices, and subsequently on the accessibility of developing countries to the global wheat stock. Wheat utilization is expected to exceed the anticipated higher production levels. In addition to that, the volume of exports will remain large, resulting in reduced world wheat stocks (15).

TO INCREASE wheat production, the application of a steady stream of new ideas to agriculture is of much more significance than the cultivation of more land (91). One example is the introduction of short-statured wheats developed by the International Maize and Wheat Improvement Centre (CIMMYT) into India, which converted this country from a wheat importer to an exporter (15,91), a phenomenon later recalled as the 'Green Revolu-

tion'. CIMMYT breeder Norman Borlaug was awarded the Nobel Peace Prize for this achievement. Another example is the effect of mechanization. Nowadays harvesting one ha. of wheat requires 1-1.5 h, whereas it took 24 hours 30 years ago. The introduction of modern technology is, however, also an economic and a cultural issue. For instance, fertilizers increase yields but their distribution and use are largely dependent on infrastructure and government subsidies. They are expensive and beneficial for modern varieties, but cause lodging in traditional landraces.

MYCOSPHEARELLA GRAMINICOLA

SEPTORIA TRITICI leaf blotch is caused by the ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt. *in* Cohn (anamorph: *Septoria tritici* Rob. ex Desm.). The description of *S. tritici* was made by Roberge and published by Desmazières in 1842 (24), and the description of *Sphaerella graminicola* was published by Fuckel in 1865. Another taxonomist, Schröter placed the latter species in the genus *Mycosphaerella* in 1894. By that time there was no functional understanding about the relation between the two fungal forms, which lasted until 1972 when Sanderson published that *M. graminicola* was the ascogenous state of *S. tritici*. Since then the wheat-*M. graminicola* pathosystem received more attention. Prior to that period a few publications dealing with *M. graminicola* were published. Weber (136) and Hilu and Bever (50) were the pioneers with regard to aspects of the pathosystem that are also dealt with in this thesis.

WHEAT AND *MYCOSPHEARELLA GRAMINICOLA*

THE IMPACT of biotic and abiotic constraints to wheat production varies widely, but annual yield losses ranging from 30% to 50% occur in countries where the production environments are poorly controlled. Improvement of production environments remains, therefore, an important aspect of wheat cultivation.

THE MAJOR biotic constraints to wheat production comprise parasitic fungi, insects and weeds. Fungal pathogens such as the bunts and smuts were of major importance, whereas the control of some rusts still requires a continuous alertness. Other diseases, such as septoria tritici leaf blotch, received relatively little attention despite the serious losses it caused, particuarly in developing countries.

SYMPTOMS OF the disease are necrotic blotches that coalesce over time, and which are filled with black fructifications. These can be either the asexual pycnidia or the sexual pseudothecia. The asexual pycnidiospores are splash-dispersed, whereas the sexual ascospores are air-borne. The dispersal of these propagules is fairly well understood, but their relative contribution to epidemiological progress is still unclear (118,119). A fact is that pycnidiospores are far less dispersed than ascospores.

TRADITIONAL HOT-SPOTS of the disease are Mediterranean, East-African, Latin-

American and Meso-American countries with a warm and humid climate (131). Here yield losses of 50% and higher have been reported. The incidence of septoria tritici leaf blotch is usually very high, which is partly due to the continuous character of wheat cultivation. Hence, crop debris carrying asexual and sexual fruiting bodies is present in the field during the planting and development of a new crop, facilitating the carry-over of septoria tritici leaf blotch. In addition to that, breeding efforts were largely frustrated by a lack of knowledge with respect to the generation, distribution and extent of genetic variation and of the resistance mechanism in wheat.

RECENTLY SEPTORIA tritici leaf blotch is also of increasing importance in regions with a temperate climate, including North-Western Europe (19, 100). The increase is partly due to an improved control of other foliar diseases, but mainly to altered cultural practices in wheat cultivation. In the past, soils were ploughed immediately after harvest, which improved the decay of the stubble, and hampered the discharge of ascospores from the pseudothecia present on it. Nowadays, due to economic motives, farmers tend to leave the fields fallow after harvest, sometimes plant a green crop without ploughing, and plant the next crop a few months later. Hence, newly planted wheat crops are much more prone to ascospore flights in autumn and winter. This is particularly demonstrated by reduced incidence of the disease in late planted wheat crops (R.A. Daamen, IPO-DLO, pers. comm.). Although cultural practices

may help to reduce the incidence of the disease, to effectively control septoria tritici leaf blotch, a thorough understanding of the wheat-*M. graminicola* pathosystem is required.

A MOST important publication on the interaction between the pathogen and the host was published in 1973 by Eyal et al. (31). In this report the authors suggested that the relationship between wheat and *M. graminicola* accorded with the gene-for-gene hypothesis developed by Flor (36,37). In this hypothesis a locus in the host is supposed to govern either a resistant or a susceptible response, and a corresponding locus in the pathogen is assumed to control either a virulent or an avirulent response. This relationship has been shown of wide validity to the genetics of many plant-pathogen interactions, particularly those involving biotrophic pathogens such as the rusts (86). The hypothesis was successfully applied in research that led to the first isolation of a fungal avirulence gene (25). The suggestion of Eyal et al. (31) was sceptically received. Since then other studies have produced conflicting evidence with respect to specificity, thus complying with a gene-for-gene system, in the wheat-*M. graminicola* pathosystem. Hence, a controversy lasted.

THIS, OF course, hampers the development of control strategies, including breeding for resistance, which is particularly serious with respect to the increasing importance of the disease. Daamen and Stol (19) described the increasing prevalence in The Netherlands during 1974-1986.

Only since 1984, wheat cultivars have been rated for 'leaf blotch' in the Dutch Recommended List of Varieties. Initial resistance scores of the majority of cultivars rarely exceed qualifications such as 'moderate', and usually decrease over time. Ratings on the 3-9 scale dropped at least one point in four years for cultivars 'Saiga' (7→5.5), 'Nautica' (6→5) and 'Estica' (6.5→5.5). Others, such as 'Arminda' and 'Okapi' remained fairly constant over at least a decade.

SANDERSONS (108) FINDING that a *Mycosphaerella* species was the ascogenous state of *S. tritici* emphasized the possible role of airborne inoculum in the establishment of the disease (19,109,118), which was also suggested in molecular studies (85).

ELUCIDATION OF the aforementioned controversy with respect to the presence and importance of specificity in the wheat-*M. graminicola* pathosystem will contribute to an enhanced perception of this pathosystem. This should also comprise an improved understanding of the resistance mechanism in wheat and of the mating system in *M. graminicola* in order to provide a firm foundation for effective control strategies.

OBJECTIVES AND APPROACH

THEREFORE FIVE major questions had to be answered. These are :

i) What is the extent of genetic variation for virulence in *M. graminicola* to wheat ?
ii) What is the extent of genetic variation for resistance in wheat ?
iii) Does specificity occur in the relationship between wheat and *M. graminicola*, and what is the relevance of it ?
iv) What is the sexual mechanism of *M. graminicola*, and how does it contribute to the generation of genetic variation ?
v) How does *M. graminicola* infect and colonize a susceptible wheat plant, and how do resistant plants defend themselves ?

THE APPROACHES undertaken to answer these questions involved inoculation experiments, using many host and pathogen genotypes, to investigate the extent of genetic variation in the fungus. Due to the characteristics of the pathogen, many replications were performed and various statistical techniques had to be applied to obtain insight in this host-pathogen relationship. Experiments were conducted in growth rooms under controlled conditions as well as in the field. To understand the contribution of the ascogenous state to the genetic variation in the pathogen, a procedure to cross isolates of the fungus was developed and the progenies were analysed using molecular techniques. Histological time-sequence studies were performed applying light microscopy, scanning and transmission electron microscopy and biochemical techniques to elucidate the pathogenesis of *M. graminicola* in wheat.

OUTLINE OF THE THESIS

THE THESIS consists of three parts. Part

one contains three chapters and describes the genetic variation for virulence and resistance in the wheat-*M. graminicola* pathosystem. The first chapter describes the occurrence of interactions between host and pathogen genotypes. The second chapter describes how to analyse such observations, and the third chapter concentrates on the detection, importance and implications of these interactions under field conditions.

PART TWO describes the mating system of *M. graminicola* and the role of genetic exchange between isolates of the pathogen and how such recombinants may contribute to disease progress.
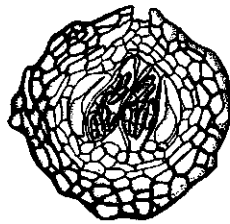
FINALLY, THE third part describes the histopathology of compatible and incompatible interactions in the wheat-*M. graminicola* pathosystem.

GENETIC VARIATION FOR VIRULENCE AND RESISTANCE IN THE WHEAT-*MYCOSPHAERELLA GRAMINICOLA* PATHOSYSTEM

CHAPTER ONE

INTERACTIONS BETWEEN
PATHOGEN ISOLATES
AND
HOST CULTIVARS

ABSTRACT

Genetic variation for virulence in 63 *Mycosphaerella graminicola* isolates, originating from 13 countries, was studied in two seedling experiments. Each experiment was performed according to a partially balanced incomplete block design with four replications over time. The first experiment put emphasis on *M. graminicola* isolates which originated from bread wheat, and comprised 50 isolates, that were inoculated on a set of testers containing 19 bread wheat cultivars, four durum wheat cultivars, and one triticale cultivar. In the second experiment more attention was paid to *M. graminicola* isolates that originated from durum wheat, and comprised 15 isolates, that were inoculated on a set of testers containing 17 durum wheat cultivars, four bread wheat cultivars, one triticale cultivar, and a *Triticum turgidum* ssp. *dicoccoides* accession. Two disease parameters, the presence of necrosis (N) and pycnidia (P) estimated as percentages of primary leaves, were employed to measure disease severity. Genetic variation for virulence in the pathogen isolates and genetic variation for resistance in the host cultivars was estimated by analyses of covariance. The significance of cultivar x isolate interactions in both experiments and for each disease parameter suggests a gene-for-gene interaction between resistance and virulence loci in host and pathogen, respectively. An agglomerative hierarchical clustering procedure, which used one *df* components of interaction between isolates and cultivars as a proximity measure, was employed to study the similarity between isolates and cultivars. Discrepancies between N and P resulted in non-identical clusters of isolates and cultivars when considering these parameters separately, which suggests that N and P are under different genetical control. Evidently, isolates of *M. graminicola* were specialized to either bread wheat or durum wheat. This was particularly evident when considering P. It is proposed, therefore, to designate two varieties in *M. graminicola*, which refer to the host species specialization in this pathogen.

Septoria tritici leaf blotch is a fungal disease of bread wheat and durum wheat, *Triticum aestivum* L. and *T. turgidum* (L.) Thell. ssp. *durum* L., respectively. The disease is caused by *Septoria tritici* Rob. ex Desm. or its teleomorph *Mycosphaerella gra-minicola* (Fuckel) J. Schröt. *in* Cohn, which has been reported in several wheat producing areas of the world (40,108,113). It is particularly a major problem in regions characterized by a temperate high rainfall environment during the wheat growing season, such as the Mediterranean Basin, Eastern and Central Africa and the Southern Cone of South America (33,67,131). High incidences and disease severities were also reported in the USA and Mexico, as well as in some European countries, New Zealand and Australia (33,100).

Relative humidity (RH) and temperature are considered to be key determinants for successful penetration of the host and its further colonization by the fungus (49). Based on interactions between temperature and leaf wetness periods, temperatures of 20-25°C were considered to promote infection (80). Pycnidia are produced under a RH

range of 35-100% with an optimum at 85% (92), although Shaw and Royle (119) reported that for a susceptible cultivar, non-conducive weather conditions in the field did not seem to limit disease establishment.

Restriction fragment length polymorphism markers (RFLPs) showed extensive genetic variation in *M. graminicola* (81,82). Genetic variation for virulence, as expressed by interactions between host and pathogen genotypes, has been a questionable subject since physiologic specialization in this fungus was suggested, despite reported declines in effectiveness and inconsistent expression of resistance in wheat to *M. graminicola* (31,33,56,88,103,106, 134). The majority of studies on the wheat - *M. graminicola* pathosystem dealt with bread wheat and bread wheat derived isolates (67,88). Tetraploid wheat species were reported to be more resistant to *M. graminicola* than bread wheat (12,138). Van Ginkel and Scharen (132,133,134) analyzed the resistance in durum wheat, and concluded that interactions between host and pathogen genotypes were of minor importance, since additive gene effects and general combining ability explained the greater part of the genetic variation for resistance that was revealed in their inheritance studies. Therefore, they suggested that host species specialization in *M. graminicola* was a much simpler explanation for reported physiologic specialization (31), and considered the absence of differential gene-for-gene relationships, thus variation for aggressiveness rather than virulence among *M. graminicola* isolates. In addition to that, proportions of the total variance in analyses of variance that were attributable to interaction were low, and not always significant (33,134,138). This ambiguity and inconclusive reports on host-pathogen interactions, obviously thwart the development of effective breeding strategies, which has been the most widely adopted strategy to control *M. graminicola* (67,88,131). Inheritance of resistance in wheat to *M. graminicola* was reported to be conditioned by single or multiple dominant and recessive genes with major effects, as well as by additivity of resistance factors with a less pronounced effect (88,133).

The present contribution is part of a larger study that was undertaken to elucidate genetic variation for virulence in the wheat-*M. graminicola* pathosystem, and comprises two experiments. The first experiment largely dealt with isolates from bread wheat, whereas the second experiment mainly involved isolates which originated from durum wheat.

MATERIAL AND METHODS

PLANT MATERIALS. In the first experiment 19 bread wheat cultivars, four durum wheat cultivars, and one triticale (X*Triticosecale* Wittmack) cultivar were employed. In the second experiment 17 durum wheat cultivars, one *Triticum turgidum* (L.) Thell. ssp. *dicoccoides* (Körn) Thell. (AABB, $2n=28$) accession, one triticale and four bread wheat cultivars were used to study genetic variation for virulence and resistance (Table 1).

M. GRAMINICOLA ISOLATES. Leaf sam-

TABLE 1. Experimental code and origin of each bread wheat and durum wheat cultivar, of an accession of *T. turgidum* ssp. *dicoccoides,* and a triticale cultivar employed to study genetic variation for virulence in *Mycosphaerella graminicola*

EC[a]	Cultivars	Origin	EC	Cultivars	Origin
Bread wheat					
An	Anza	Algeria	KT	Klein Titan	Argentina
Ar	Arminda	The Netherlands	KU	Kavkaz/UP301	Mexico
BL	Beth Lehem	Israel	KZ	Kavkaz	USSR
Bo	Bobwhite	Mexico	La	Lakhish	Israel
Ce	Ceeon	Israel	Ob	Obelisk	The Netherlands
Co	Colotana	Brazil	Ol	Olaf	USA
Ge	Gerek 79	Turkey	Sh	Shafir	Israel
Ia	Iassul 20	Brazil	T29	Taichung 29	Japan
K7	Kavkaz/7C	Mexico	To	Toropi	Brazil
KK	Kavkaz/K4500 l.6.a.4	Mexico	Ve	Veranopolis	Brazil
Durum wheat					
A65	Acsad 65	Algeria	Ma	Marzak	Tunisia
B17	Bidi 17	Algeria	MB	M.B. Bachir	Algeria
BD	BD2777	Morocco	OR	Omrabi 5	Morocco
Ca	Cakmak 79	Turkey	OZ	OZ 368	Algeria
Cc	Cocorit	Morocco	Sa	Safir	Tunisia
Et	Etit 38	Israel	Te	Tensift	Morocco
H3	Hedba 3	Algeria	Vo	Volcani 447	Israel
I69	Inrat 69	Algeria	Wa	Waha	Algeria
In	Inbar	Israel	ZB	Zenati Bouteille	Algeria
Jo	Jori	Morocco	ZP	ZB/*T. polonicum*	Algeria
T. turgidum ssp. *dicoccoides*					
G25	G25	Israel			
Triticale					
Be	Beagle	Mexico			

[a]EC:Experimental code.

ples were collected in 13 countries and originated from bread wheat and durum wheat cultivars. Monopycnidial *M. graminicola* isolates were obtained (60), which were used to inoculate susceptible wheat cultivars (cvs. Inbar and Lakhish for durum wheat and bread wheat isolates, respectively). Desiccated colonized primary leaves were stored for short term preservation. Sixty-three isolates were selected on a regional basis, hence most countries were represented by accessions from several more or less distant locations (Table 2). Inoculum was prepared by inoculating 50 ml liquid yeast-glucose medium, in 100 ml erlenmeyer flasks, with fresh *M. graminicola* colonies from agar plates. For each experiment, two flasks per

isolate were incubated for five days in a temperature controlled reciprocal shaker at 15°C. The resultant spore suspensions were pelleted by centrifuging at 10,000 rpm for 10 min, resuspended in de-ionized water, and adjusted to a density of 10^7 spores/ml.

EXPERIMENTAL DESIGN. The experiments comprised sets of inoculations and were conducted according to a partially balanced incomplete block design with respect to pathogen isolates, which permitted the execution of four replicates over time. Experiment 1 involved 25 blocks of eight

TABLE 2. Experimental code and origin of 63 *Mycosphaerella graminicola* isolates studied for genetic variation of virulence towards 23 wheat cultivars and one triticale cultivar

EC[a]	Isolate	Country	Location	EC	Isolate	Country	Location
AR1	IPO86063	Argentina	Balcarce	BU2	IPO88024	Burundi	Tora
AR2	IPO86068	Argentina	Balcarce	RW1	IPO88037	Rwanda	Tamira
AR3	IPO87022	Argentina	Pergamino	UG1	IPO88038	Uganda	Kalengyere
AR4	IPO87023	Argentina	Pergamino	NL1	IPO235	Netherlands	Anjum
AR5	IPO87024	Argentina	Pergamino	NL2	IPO89011	Netherlands	Barendrecht
AR6	IPO86078	Argentina	Tres Arroyos	NL3	IPO89013	Netherlands	Drenthe
UR1	IPO87019	Uruguay	Colonia	NL4	IPO88025	Netherlands	Ebelsheerd
UR2	IPO87021	Uruguay	Colonia	NL5	IPO89012	Netherlands	Wageningen
UR3	IPO87016	Uruguay	Dolores	NL6	IPO89010	Netherlands	Zelder
UR4	IPO87018	Uruguay	Dolores	TK1	IPO86013	Turkey	Adana
UR5	IPO87020	Uruguay	Dolores	TK2	IPO88014	Turkey	Adana
ET1	IPO88005	Ethiopia	Assassa	TK3	IPO88015	Turkey	Adana
ET2	IPO88010	Ethiopia	Bekoje	TK4	IPO88016	Turkey	Adana
ET3	IPO88012[b]	Ethiopia	Bekoje	TK5	IPO86022[b]	Turkey	Altinova
ET4	IPO88020	Ethiopia	Bekoje	TK6	IPO86023	Turkey	Altinova
ET5	IPO88013	Ethiopia	Blue Nile Valley	TK7	IPO86010	Turkey	Tasci
ET6	IPO88018	Ethiopia	Holetta	TK8	IPO86009	Turkey	Tasci
ET7	IPO88019	Ethiopia	Holetta	TK9	IPO86008	Turkey	Tasci
ET8	IPO88021	Ethiopia	Holetta	TK10	IPO88017	Turkey	unknown
ET9	IPO88004	Ethiopia	Kulumsa	AL1	IPO90020	Algeria	Guelma
ET10	IPO88022	Ethiopia	Mota	TN1	IPO91009[b]	Tunisia	Beja
ET11	IPO88027	Ethiopia	Sinana	TN2	IPO91010[b]	Tunisia	Beja
KE1	IPO87000[c]	Kenya	Eldoret	TN3	IPO91016[b]	Tunisia	Beja
KE2	IPO87011[c]	Kenya	Eldoret	TN4	IPO91011[b]	Tunisia	Tunis
KE3	IPO87008	Kenya	Eldoret	TN5	IPO91012[b]	Tunisia	Sidi Ncir
KE4	IPO87015	Kenya	Mou Narok	TN6	IPO91014[b]	Tunisia	Mateur
KE5	IPO87009	Kenya	Njoro	TN7	IPO91015[b]	Tunisia	Fetissa
KE6	IPO87012	Kenya	Njoro	SY1	IPO91004[b]	Syria	Lattakia
KE7	IPO87013	Kenya	Njoro	MO1	IPO91017[b]	Morocco	O. Frej
KE8	IPO86026	Kenya	Timau	MO2	IPO91018[b]	Morocco	J. Shaim
BU1	IPO88023	Burundi	Tora	MO3	IPO91019[b]	Morocco	Meknes
				MO4	IPO91020[b]	Morocco	Doukhala

[a]EC:experimental code.
[b]Collected from durum wheat.
[c]Collected from the same sample.

main plots (=isolates), and experiment 2 comprised 10 blocks of six main plots (11). The host cultivars were randomly allocated to 24 subplots in each main plot.

EXPERIMENTAL PROCEDURES AND CONDITIONS. Ten to 15 seeds per accession were linearly sown in 5x5 cm plastic pots with a peat/sand mixture. Plants were grown in controlled walk-in climate chambers with similar pre- and post inoculation conditions with respect to light intensity and day length (56 μE.sec^{-1}.m^{-2} for 16 h.day^{-1}). Pre- and post-inoculation temperature and RH conditions were 18/16°C (day/night rhythm) and 70% RH, and 22/21°C and ≥85% RH, respectively.

Quantitative inoculations were conducted by spraying spore suspensions, 30 ml/isolate supplemented with two drops of Tween 20® surfactant, on the test cultivars that were randomized on a turn table, adjusted at 15 rpm, in a closed inoculation cabinet equiped with interchangeable atomizers and a water cleaning device to avoid contamination. Incubation was conducted under polyethylene covered aluminum frames, providing leaf wetness for 48 h at a light intensity of approximately 3 μE.sec^{-1}.m^{-2}. Fertilizer (Sporumix PG®, 0.5 g.l^{-1}) was applied at seven days after inoculation, and the emerging second leaves were clipped 14 days after inoculation in order to facilitate light penetration to the primary leaves and disease assessment. Disease severity was evaluated at 21 days after inoculation using two parameters; the presence of necrosis (N) and pycnidia (P) estimated as percentages of the total primary leaf area of individual

seedlings. These values were averaged per pot for further analyses of the disease parameters N and P.

DATA ANALYSES. The experimental design was only partially balanced, which would imply that the corresponding statistical regression model would comprise more than a thousand parameters. This was computationally not feasible, hence it appeared appropriate to subject the responses N and P to analyses of covariance (ANCOVAs). Block differences were fully accounted for by setting one covariate for each block. Since blocks were parts of the experiment that were carried out sequentially, block effects were confounded with possible time effects. These effects could only be partly disentangled, hence block effects were used to adjust the responses. A consequence of this procedure is that tables of means may contain some negative values, after adjusting for covariates (Tables 3-6). Statistical analyses were conducted using the Genstat 5 package (41) on transformed (arc-sin) and untransformed data sets. Since transformations did not substantially stabilize the residual variance and did not influence the conclusions, untransformed data are presented here.

In order to reveal structures of the interactions between host and pathogen genotypes, the tables of means were subjected to a hierarchical agglomerative clustering procedure as described by Corsten and Denis (18). The procedure performs grouping of rows and columns in the tables as to identify a minimum number of groups that account for the overall interaction.

The groups are internally homogeneous. In each step of this sequential procedure, the mean square for interaction (MS_{int}) is calculated for all possible subtables consisting of a pair of rows or a pair of columns of the full table. The pair of rows or columns with minimal MS_{int} is merged, giving an updated table, and the process is repeated. Thus, a sequence of amalgamations of rows and columns is produced, eventually leading to a two-by-two table. In this way the total sum of squares for interaction (S) is built up from orthogonal increments, each connected with a merge as described, to obtain insight in a possible structure of the interaction. Corsten and Denis (18) formulated an F test procedure to stop clustering just before S exceeds the critical value $c(x) = ns^2F(n, f, \alpha)$, where n = (number of isolates-1) x (number of cultivars-1), s^2 = an estimate of the residual variance obtained independently from the two-way tables subjected to the cluster analysis, and $F(n, f, \alpha)$ = the upper α point of the F distribution with n and f degrees of freedom. This procedure determines the probability of stopping too early, *i.e.* of ending up with too many groups, under the H_0 hypothesis of no interaction between isolates and cultivars. Thus, it determines which isolates or cultivars are significantly different from each other.

RESULTS

SYMPTOM DEVELOPMENT. The inoculated leaves remained green during the first eight to nine days after inoculation. The first symptoms were generally observed as necrosis, starting at the leaf tips. In susceptible cultivars, necrotic blotches quickly coalesced, which eventually resulted in high N levels, with maxima of 90% in experiment 1 and 100% in experiment 2 at 21 days after inoculation. Necrosis was generally straw-coloured, though characteristic straw-reddish to greyish-black phenotypes were observed in responses with few and abundant pycnidia, respectively. Pycnidia were almost entirely produced in the necrotic area, and appeared somewhat later than the necrosis. In very susceptible responses, pycnidia sometimes occurred in still green, but collapsing tissue. Resistance was expressed as low P, which was not necessarily concurrent with N (Tables 3-6, data adjusted for block effects). Low N was sometimes confined to small necrotic spots, which resembled those developed in hypersensitive responses towards obligate parasites such as the cereal rusts and powdery mildews, that were particularly evident in the responses of bread wheat cultivars towards durum wheat derived isolates.

Bread wheat derived isolates almost exclusively produced pycnidia in the bread wheat cultivars, whereas pycnidial production by durum wheat derived isolates was almost entirely restricted to the durum wheat cultivars. A few cultivars, particularly cv. Inbar, allowed some pycnidia production of isolates that originated from both wheat species (Tables 4 and 6). The discrimination between isolates that were derived from either bread wheat or durum wheat was less evident when considering N (Tables 3 and 5, Figs. 1-4), since bread wheat isolates usually induce abundant

necrosis in the durum wheat cultivars, whereas durum wheat isolates generally cause little necrosis in the bread wheat and triticale cultivars (Tables 3 and 5). Isolates *IPO88012-ET3* (see Tables 3 and 4), *IPO91010-TN2* and *IPO91019-MO3* (see Tables 5 and 6) were derived from durum wheat, but appeared to be adapted to bread wheat, as evidenced by high *P* levels in bread wheat, but low *P* levels in durum wheat. *N* levels were high in both species. A few durum wheat cultivars, such as 'Inbar' and 'Omrabi 5', allowed some pycnidia formation whereas the triticale cv. Beagle was not affected by either type of *M. graminicola* isolate (Tables 4 and 6).

INTERACTIONS BETWEEN ISOLATES AND CULTIVARS. ANCOVAs were conducted on complete and on restricted response matrices for *N* and *P* (Table 7). The restricted analyses in each experiment merely included either bread wheat cultivars and bread wheat derived isolates, or durum wheat cultivars and durum wheat derived isolates. Such analyses were necessary to eliminate any contribution to the MS_{int} of the aforementioned observation that bread wheat and durum wheat isolates almost exclusively produce pycnidia in their respective host species. The interactions between cultivars and isolates were highly significant (P<0.01) for parameters *N* and *P*. Numerous interactions were observed in the response matrices for *N* and *P* (Tables 3-6), though simultaneous consideration of these parameters revealed that they were not necessarily corresponding, so that the relationship between pathogen isolates and host

cultivars can be categorized into four types. Responses can be non-interactive for both *N* and *P*, interactive for both *N* and *P*, interactive for *N* and non-interactive for *P*, or *vice versa*, which suggests that *N* and *P* are under dissimilar genetic control (Table 8).

CLUSTER ANALYSES. The response matrices of *N* and *P* of both experiments were subjected to cluster analyses, which resulted in dendrograms for isolates and cultivars for each disease parameter (Figs. 1-4).

Experiment 1. The analysis for *N* resulted in 31 and 21 significantly different clusters for isolates and cultivars, respectively (Fig. 1). For *P*, the analysis resulted in 21 and 18 significantly different clusters for isolates and cultivars, respectively (Fig. 2). Each cluster, considering both *N* and *P*, contained less than five accessions. The number of significantly different clusters per country was substantial. For example, 10 Turkish isolates (Table 2) were attributed to nine significantly different clusters for both *N* and *P*. Similar comparisons for the other countries indicate extensive genetic variation for virulence not only between but also within local populations of the fungus, since most countries were represented by multiple accessions from several locations (Table 2). For example, 11 isolates were sampled at seven locations in Ethiopia, which were assigned to seven significantly different clusters for *N*, though the isolate from Assassa (*IPO88005-ET1*) was merged with the three isolates from Bekoje, representing the central southern part of the country. The three isolates from Holetta were

TABLE 3. Adjusted necrosis (*N*) response matrix of experiment 1; 24 host accessions and 50 *Mycosphaerella graminicola* isolates, arranged according to the clusters of Fig. 1[a]

EC[c]	Cultivars[b]																							
	B La	B Sh	B Co	B To	B Ge	B Ia	B Ce	D Vo	D Et	B BL	D Ca	T Be	B Bo	D In	B T29	B KT	B Ol	B Ob	B Ar	B Ve	B KK	B KU	B KZ	B K7
TK3	69	64	49	56	76	50	55	45	64	57	49	30	22	63	84	83	60	51	67	40	20	13	29	21
TK4	78	57	62	64	78	55	67	41	61	52	58	35	25	63	83	87	58	59	52	55	20	7	22	27
UR5	59	71	53	66	77	54	63	50	43	47	57	18	39	69	71	79	72	62	33	49	15	9	23	12
UR3	51	67	32	47	65	56	58	30	45	43	58	26	26	54	79	79	54	36	24	19	5	5	16	14
ET11	44	38	39	31	72	27	28	38	51	18	34	18	6	35	77	67	29	49	48	23	5	6	3	23
AR6	50	57	28	54	75	48	54	44	16	49	48	7	10	52	72	76	53	54	50	69	1	1	10	6
AR3	62	72	21	32	82	65	65	31	32	44	44	7	9	5	82	81	56	56	53	68	-1[d]	0	8	5
AR4	59	69	41	36	80	57	62	25	42	38	49	24	27	5	74	79	63	43	49	52	1	0	8	18
UR2	42	64	31	38	77	52	57	29	45	34	45	16	21	19	81	88	56	71	58	40	12	6	5	17
TK9	71	73	30	30	83	35	63	21	26	21	53	18	12	18	90	85	49	35	38	38	7	9	11	13
AR1	53	71	64	60	79	58	63	41	54	43	61	57	38	5	76	80	60	51	44	38	13	7	5	24
TK6	47	61	58	53	79	42	27	46	34	37	60	10	15	25	74	83	60	53	21	45	8	6	11	9
UR1	54	66	30	48	82	37	50	35	20	38	50	10	40	18	87	86	69	29	14	11	3	9	12	10
ET5	46	67	52	63	77	52	31	34	50	28	42	13	13	42	43	23	16	21	11	17	15	15	20	13
ET10	55	67	58	79	79	58	48	67	47	35	55	31	14	64	78	55	8	64	17	74	19	10	26	19
TK5[e]	7	14	47	15	33	29	2	68	71	17	70	23	14	61	54	28	7	10	3	41	5	9	8	10
NL5	24	33	41	50	23	22	21	11	16	25	35	6	22	33	75	79	29	56	43	17	18	19	54	5
NL1	38	46	48	55	25	26	43	23	44	32	59	13	33	42	85	84	49	67	57	32	34	28	73	10
ET3[e]	68	62	44	69	69	52	42	41	37	34	50	9	34	40	80	75	39	69	35	52	51	40	61	60
ET2	65	69	66	69	77	52	56	44	50	36	55	30	34	55	80	84	51	81	56	50	58	50	72	65
ET4	49	62	36	65	73	47	21	32	22	30	50	9	22	43	78	76	47	68	26	46	29	37	49	51
ET1	72	72	62	87	86	57	54	50	40	47	59	27	37	69	89	82	63	85	76	69	34	45	70	83
ET7	58	64	65	64	71	62	45	48	51	32	63	0	33	56	78	79	57	54	45	73	37	58	71	73
NL2	43	55	50	76	43	42	41	37	15	31	50	29	42	57	84	84	58	71	65	69	36	47	81	68
BU2	59	69	69	74	75	74	59	24	35	37	50	26	7	50	83	75	65	65	60	73	28	25	46	55
TK1	66	66	49	67	65	47	66	27	47	44	49	13	12	33	80	81	46	68	65	51	5	21	30	42
NL4	45	36	35	15	78	58	31	21	53	31	38	-5	13	48	78	78	37	62	60	-4	26	42	43	56
NL6	48	57	42	80	78	35	47	27	56	42	62	24	43	80	88	87	52	82	75	14	36	61	87	67
NL3	6	14	33	4	81	57	60	28	44	11	36	-1	2	13	71	39	5	85	83	0	0	6	38	7
UR4	52	68	55	62	74	34	52	14	19	44	47	11	17	39	62	79	47	53	30	5	14	41	61	51
KE4	53	56	56	71	62	27	45	28	15	33	48	6	26	44	60	63	63	59	31	5	22	34	63	48
KE5	57	61	41	62	77	40	41	27	19	23	51	2	24	34	70	86	51	51	38	7	10	32	74	58
KE2	57	73	52	65	77	48	59	41	25	40	43	8	28	48	85	75	71	71	37	32	42	41	73	80
KE6	49	68	58	80	71	58	48	34	22	51	50	6	11	56	71	78	61	55	34	-6	24	14	58	69
KE8	75	74	65	85	86	55	70	26	35	39	74	22	21	64	85	91	56	66	39	11	25	38	61	62
RW1	47	71	66	88	69	32	61	16	24	39	48	9	25	66	86	86	60	62	12	10	17	56	81	69
KE7	61	66	66	75	70	46	55	21	50	42	57	28	22	59	69	71	48	53	28	10	29	31	71	62
KE1	63	70	66	80	82	53	57	38	39	47	57	41	39	52	76	80	66	46	30	8	26	29	66	64
UG1	68	68	61	70	74	37	55	28	57	31	54	23	28	41	80	76	67	56	23	10	27	52	63	69
TK2	81	67	66	75	77	47	50	31	47	45	52	25	36	43	81	76	62	62	32	42	26	26	51	56
BU1	65	69	56	63	80	38	62	24	35	34	35	18	26	45	81	72	55	43	20	34	10	30	56	61
KE3	64	74	59	78	86	35	50	21	53	36	61	25	15	41	61	74	49	60	26	17	20	25	56	25
ET8	69	78	68	78	80	45	73	45	59	39	59	32	47	71	87	80	59	64	15	39	66	51	70	79
ET6	52	56	54	78	60	20	46	59	34	32	54	9	34	55	73	75	48	57	13	10	30	44	71	61
TK7	67	53	38	62	69	49	55	56	44	51	60	24	23	61	75	69	54	42	22	45	33	33	66	60
TK10	51	51	54	62	69	38	38	47	39	42	60	25	14	54	80	42	7	74	10	51	64	33	71	59
TK8	64	53	41	73	70	55	39	60	58	53	63	27	18	59	72	81	12	69	29	16	31	40	73	57
ET9	55	79	59	75	84	56	59	60	37	36	43	14	16	46	66	52	13	29	10	15	63	46	58	70
AR2	67	64	50	60	71	56	61	62	42	48	63	53	38	30	75	79	59	59	29	28	67	46	59	64
AR5	79	80	41	51	75	38	55	74	32	50	42	28	62	32	86	75	70	60	26	12	68	68	67	73

[a] $LSD_{0.01}$=29, $LSD_{0.05}$=22.
[b] Experimental codes for cultivars according to Table 1; B: bread wheat, D: durum wheat, T: triticale.
[c] Experimental codes for isolates according to Table 2.
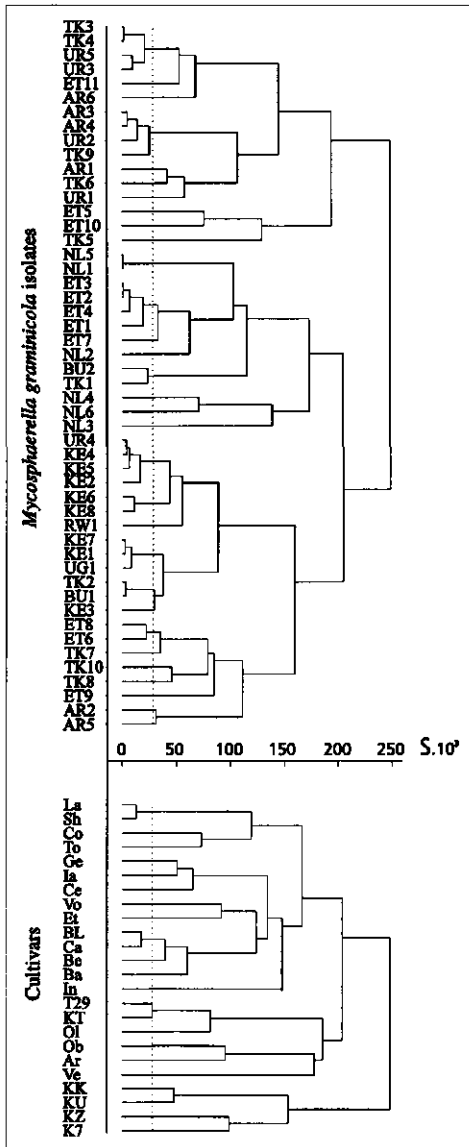[d] Negative values are due to block adjustments.

Fig. 1. Dendrograms of simultaneously clustered genotypes of wheat (23) and triticale (1), and *Mycosphaerella graminicola* isolates (50), based on N in experiment 1. The positions of the nodes correspond with the cumulative sum of squares for interaction between cultivars and isolates (S) on the horizontal axis. The area at the left of the vertical dotted line represents non-significant differences at P=0.05, S=28.12*10³ (for P=0.01, S=29.78*10³).

placed in two clusters for *N*. For *P*, only four isolates were clustered, one cluster comprised two isolates from Bekoje (*IPO88010-ET2* and *IPO88012-ET3*) and the other cluster combined an isolate from Bekoje and the isolate from Assassa (*IPO88020-ET4* and *IPO-88005-ET1*). The remaining seven isolates were significantly different at P=0.01, thus not clustered, which implies considerable genetic variation for virulence within a location such as Holetta, where the cvs. Colotana, Kavkaz/K4500 l.6.a.4 and Veranopolis differentiated the isolates. Analogous comparisons revealed similar results for isolates from other countries. The Kenyan isolates showed the least variation, though two isolates from El-doret, which were derived from the same leaf sample (*IPO87000-KE1* and *IPO87011-KE2*), proved to be significantly different, particularly for *P*.

Isolate *IPO89012-NL5* was isolated from a field plot that was inoculated with *IPO235-NL1*, hence these two isolates were considered to be similar, as reflected in the cluster analysis, which clustered them in the first step for *N*, and in the fifth step for *P*.

Dissimilarities among the cultivars were particularly evident in the dendrogram for *P* (Fig. 2). The durum wheat accessions were in one cluster, as were cv. Kavkaz and one of its derivatives, 'Kavkaz/UP301'. A third cluster comprised the cvs. Iassul 20, Beagle and Bet Lehem, which were very resistant to most isolates. The majority of the cultivars, however, was not clustered, for *P* and *N*, indicating considerable genetic variation for resistance to *M. graminicola* in the tested cultivars.

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TABLE 4. Adjusted pycnidia (P) response matrix of experiment 1; 24 host accessions and 50 *Mycosphaerella graminicola* isolates, arranged according to the clusters of Fig. 2[a]

EC[c]	Cultivars[b]																							
	D Vo	D Et	D Ca	D In	B KZ	B KU	B K7	B Co	B To	B Ia	T Be	B BL	B Ar	B Bo	B KK	B Ve	B Ob	B T29	B La	B Ce	B Ge	B Sh	B KT	B Ol
TK5[e]	53	50	41	40	-2[d]	-2	-2	-2	-1	-2	-2	-2	-2	-2	-2	11	-2	0	-2	-2	-2	-2	0	-1
NL5	1	1	1	1	8	4	3	1	2	1	1	1	4	5	1	5	8	25	11	9	1	2	30	6
NL1	-1	-1	-1	0	8	0	0	-1	2	-1	-1	0	7	3	-1	7	10	21	20	14	-1	12	20	18
BU2	1	1	1	1	8	5	8	12	20	6	1	4	5	1	1	25	15	23	21	23	35	21	27	17
ET11	0	0	1	3	0	0	0	2	4	0	0	0	10	0	0	1	26	34	32	17	36	25	27	14
NL4	-2	-2	0	-2	-2	6	8	-2	-2	-1	-2	-2	5	-1	-1	-2	32	17	17	5	16	3	45	0
NL6	0	0	0	7	14	22	26	1	21	2	0	5	7	11	1	0	44	14	20	24	35	15	35	9
NL3	-1	-1	-1	0	2	0	0	-1	0	1	-1	-1	25	-1	-1	-1	38	4	1	32	48	0	18	0
ET10	20	9	10	21	0	1	0	14	53	19	1	9	0	0	2	59	38	57	39	34	64	55	9	1
ET9	4	2	1	5	21	26	39	28	35	8	1	5	1	1	19	2	9	14	40	41	54	55	14	2
ET5	2	2	12	11	1	2	1	9	21	13	1	4	1	1	6	1	2	4	39	21	52	52	7	2
KE7	3	4	3	3	14	8	21	21	21	4	3	5	3	5	4	3	7	11	23	30	34	29	29	26
KE3	5	4	4	5	11	7	9	17	20	5	4	4	4	4	4	4	14	8	34	28	45	28	27	27
KE1	2	3	2	4	4	3	9	16	9	2	2	6	2	6	2	2	6	7	29	18	32	27	36	20
KE6	1	0	1	1	12	5	10	14	15	0	0	8	0	0	7	0	21	6	33	35	12	34	33	33
UR4	0	-1	0	0	19	11	14	12	15	0	0	1	0	0	1	0	11	16	33	32	43	37	46	33
KE4	0	0	3	0	19	10	23	20	34	0	0	4	0	1	3	0	18	14	35	31	36	36	37	45
KE8	0	0	1	1	4	11	16	22	36	0	0	5	10	2	1	0	10	22	50	40	40	47	44	35
KE5	-1	-1	-1	-1	16	12	42	19	27	1	-1	1	1	2	-1	0	20	13	43	28	39	43	64	30
ET6	10	0	5	14	23	28	39	31	32	1	0	4	0	22	4	1	14	38	41	37	39	43	38	32
UG1	0	0	0	0	19	25	44	30	36	2	0	0	0	0	6	0	9	27	36	35	38	51	34	25
RW1	0	-1	0	15	31	37	40	25	43	0	0	0	0	1	2	1	17	39	34	43	34	47	63	32
TK8	1	2	9	16	23	28	42	12	51	5	1	8	4	7	7	1	41	38	53	22	38	44	73	1
TK2	1	1	1	1	6	8	19	16	4	1	2	1	2	1	4	3	37	40	21	36	40	33	29	
BU1	-1	-4	-1	-4	19	9	10	31	33	0	-1	0	-1	7	-1	18	10	38	43	43	49	47	42	35
ET7	1	0	10	12	10	24	14	30	24	7	-1	0	11	16	0	40	4	28	51	30	39	47	40	37
ET4	0	1	7	4	11	19	36	2	26	5	0	3	2	9	3	28	51	45	34	14	29	45	37	25
ET1	0	-1	1	6	14	14	39	9	22	8	0	1	9	7	1	45	43	52	43	25	41	43	44	30
ET3[e]	10	0	2	8	11	13	24	6	29	1	0	0	6	16	9	21	51	40	51	25	46	50	58	19
ET2	0	0	4	4	19	21	26	12	16	17	0	1	4	20	22	30	39	40	54	44	43	57	52	23
TK10	0	0	3	1	14	12	47	17	14	4	0	0	0	0	30	35	53	40	22	31	58	33	0	0
NL2	-3	0	1	6	42	25	44	6	28	1	0	5	30	27	8	45	54	55	14	31	9	43	61	45
TK7	1	1	2	14	22	19	31	5	16	1	1	26	1	2	3	15	16	41	54	42	37	36	52	30
KE2	0	0	0	0	31	22	39	2	18	0	0	10	7	7	7	12	37	36	47	38	46	42	42	41
ET8	0	3	1	1	10	19	21	1	16	2	1	1	1	20	22	12	23	25	40	39	34	26	34	29
AR5	7	0	0	7	8	38	30	4	3	2	1	15	0	38	20	1	20	40	41	35	26	48	35	43
AR2	2	2	2	2	10	32	41	3	12	6	4	12	3	29	35	7	37	46	64	56	53	60	54	54
TK3	1	0	1	21	1	1	1	12	20	8	2	19	26	9	1	1	34	66	55	43	54	47	72	29
TK4	4	4	5	28	4	4	4	19	31	17	3	33	23	9	3	6	38	62	69	59	67	53	66	33
AR3	-1	-1	0	-1	-1	-1	-1	0	0	16	-1	10	27	1	-1	40	24	58	57	54	65	52	64	51
AR4	2	2	2	2	2	2	2	8	10	28	2	9	24	18	2	41	22	48	44	54	68	54	57	48
TK9	0	0	0	1	0	0	0	1	5	2	0	0	7	1	0	22	20	57	54	47	57	52	57	29
AR6	-2	-2	-2	-1	-2	-1	1	0	0	14	-2	4	6	-1	-2	16	27	12	30	36	43	42	44	27
UR2	3	6	3	8	3	3	3	5	3	12	3	4	24	8	3	13	42	41	32	41	50	50	47	30
TK1	3	1	14	16	11	8	7	11	37	9	2	13	31	2	2	32	46	33	54	57	50	49	51	33
TK6	2	2	5	4	2	2	2	14	12	10	2	6	3	9	2	28	33	44	22	15	52	51	61	54
AR1	4	3	3	3	4	4	4	4	4	3	15	5	25	3	7	12	23	26	34	45	43	39	38	
UR1	0	0	0	4	0	0	0	0	1	0	0	3	0	18	0	0	0	26	30	36	45	48	61	47
UR5	-3	-3	-3	5	-3	-3	-3	14	5	-3	-3	0	-3	15	-3	16	20	25	21	39	34	48	60	45
UR3	2	1	1	13	1	1	1	8	11	1	6	25	2	20	1	8	22	45	45	52	43	66	62	44

[a] LSD$_{0.01}$=20, LSD$_{0.05}$=15.
[b] Experimental codes for cultivars according to Table 1; B: bread wheat, D: durum wheat, T: triticale.
[c] Experimental codes for isolates according to Table 2.
[d] Negative values are due to block adjustments.
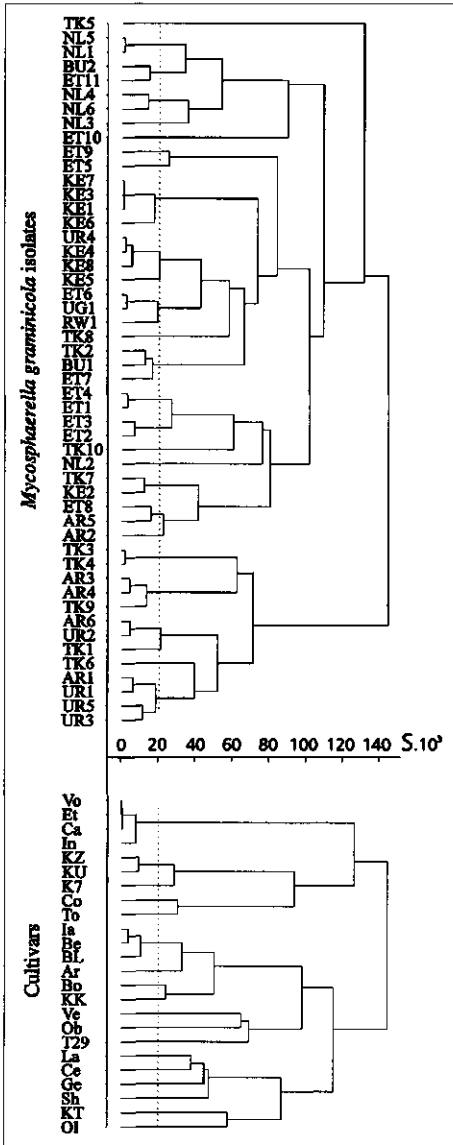[e] Durum wheat derived isolates.

Fig. 2. Dendrograms of simultaneously clustered genotypes of wheat (23) and triticale (1), and *Mycosphaerella graminicola* isolates (50), based on *P* in experiment 1. The positions of the nodes correspond with the cumulative sum of squares for interaction between cultivars and isolates (*S*) on the horizontal axis. The area at the left of the vertical dotted line represents non-significant differences at *P*=0.05, $S=20.12*10^3$ (for P=0.01, $S=21.17*10^3$).

Experiment 2. The 15 isolates were separated into eight and 10 significantly different clusters for *N* and *P*, respectively (Figs. 3 and 4). The four isolates that were particularly pathogenic on bread wheat cultivars, *IPO-88018-ET6*, *IPO90020-AL1*, *IPO91019-MO3* and *IPO91010-TN2*, were separated, for both *N* and *P*, from the remaining 11 isolates that were clearly adapted to the durum wheat cultivars. Due to the virulence differences, *IPO-88018-ET6*, *IPO90020-AL1*, *IPO91019-MO3* and *IPO91010-TN2* were placed in two significantly different clusters. Isolate *IPO91019-MO3* only produced high *P* levels in cv. Anza, whereas the others were also virulent on cv. Lakhish and to a lesser extent on cv. Bobwhite. Considering *P* of the durum wheat derived isolates, those originating from Tunisia were all significantly different from each other, which implies ample genetic variation for virulence among and within locations (e.g. Beja, isolates *IPO91009-TN1*, *IPO91010-TN2* and *IPO91016-TN3*). Considering *P*, Isolate *IPO91014-TN6* was grouped with *IPO91004-SY1* from Syria, whereas *IPO91016-TN3* showed most similarity to two Moroccan isolates (*IPO-91020-MO4* and *IPO91018-MO2*). The remaining Moroccan isolate (*IPO-91017-MO1*) was unique.

The arrangement of the cultivars over the clusters was not analogous for the two disease parameters (eight and 12 significantly different clusters for *N* and *P*, respectively; Figs. 3 and 4). For instance, cv. Marzak and *T. dicoccoides* G25 were in the same cluster for *N*, but for *P* the latter was clustered with the highly resistant bread wheat cvs. Kavkaz/K4500 l.6.a.4 and Bobwhite,

TABLE 5. Adjusted necrosis (N) response matrix of experiment 2; 23 host accessions and 15 *Myco-sphaerella graminicola* isolates, arranged according to the clusters of Fig. 3[a]

EC[c]	Cultivars[b]																						
	D I69	D ZB	D BD	D OZ	D B17	D H3	D ZP	D MB	D In	D Wa	D OR	D Te	D Ma	D G25	D Jo	D A65	D Sa	B La	B An	B Bo	B KK	D Cc	T Be
TN5	84	82	85	82	69	83	83	72	69	68	62	76	72	63	72	69	71	1	-2[d]	-1	-4	77	1
TN1	77	95	83	100	86	93	78	78	87	78	66	78	76	66	74	67	77	8	8	5	3	78	8
MO2	86	85	75	78	74	71	76	68	69	54	55	70	69	59	59	49	63	6	5	3	0	62	9
TN3	97	97	92	86	74	90	83	84	78	80	72	84	90	54	61	72	68	7	7	2	4	90	5
SY1	84	80	66	85	65	85	79	68	82	64	56	81	82	44	65	83	75	10	5	6	2	62	7
TN4	80	79	75	88	82	84	88	90	89	81	70	83	86	65	83	84	82	28	29	3	4	84	4
MO1	74	82	94	98	90	95	85	94	90	64	62	75	81	53	90	87	90	11	7	9	3	95	32
TN7	51	65	87	54	31	79	50	65	68	49	48	60	72	44	47	31	47	3	5	5	4	59	10
MO4	82	101[c]	103	98	72	95	86	80	63	47	55	79	99	57	74	52	49	11	8	6	7	83	5
TN6	85	104	83	92	78	98	80	79	58	60	36	63	86	55	66	46	47	10	9	6	6	66	8
TK5	77	73	58	42	32	33	43	29	57	59	60	67	70	56	43	35	47	11	12	14	9	71	26
MO3	18	35	19	41	16	14	20	17	13	11	8	9	43	37	11	14	37	9	62	11	8	58	6
ET6	49	55	63	60	37	53	63	56	66	69	63	76	71	64	66	51	56	68	74	52	53	80	36
TN2	48	65	44	51	28	43	54	40	87	61	71	82	70	40	60	39	52	78	91	19	26	86	23
AL1	14	31	43	42	13	32	24	15	68	68	39	57	43	21	24	24	8	75	71	37	5	80	11

[a] $LSD_{0.01}=29$, $LSD_{0.05}=20$.
[b] Experimental codes for cultivars according to Table 1; B: bread wheat, D: durum wheat, T: triticale.
[c] Experimental codes for isolates according to Table 2.
[d] Values >100 and <0 are due to block adjustments.

and with the triticale cv. Beagle, due to the low *P* levels in these accessions, and hence the absence of interactions. However, cv. Marzak was clustered with cv. Tensift, and displayed a highly susceptible response with the majority of the durum wheat derived isolates. Similarity for *P* was observed between the cvs. OZ 368, Bidi 17, Hedba 3 and M.B. Bachir, which are land races cultivated in Algeria. These cultivars occurred in one cluster for *N*, but were pairwise separated for *P*, particularly due to the responses with isolates *IPO91011*-TN4, *IPO91012*-TN5 and *IPO91015*-TN7 (see Table 6). For *P*, the bread wheat cvs. Lakhish and Anza were individually separated from cvs. Kavkaz/K4500 1.6.a.4 and Bobwhite, due to their susceptibility for isolates *IPO88018*-ET6, *IPO91010*-

TN2 and *IPO90020*-AL1, and the differential response to *IPO91019*-MO3 (see Table 6). The dendrograms of isolates and cultivars (Figs. 1-4), indicate ample genetic variation for virulence and resistance in the wheat-*M. graminicola* pathosystem. Comparison of these dendrograms reveals significant discrepancies between isolate and cultivar clusters for the two response parameters, *i.e.* entries that constitute a cluster for *N*, not necessarily form a similar cluster for *P* (see also Tables 3-6 and 8).

EFFICACY OF RESISTANCE. The bread wheat and durum wheat cultivars were grouped according to their response to the bread wheat and durum wheat derived isolates in experiments one and two, respectively.
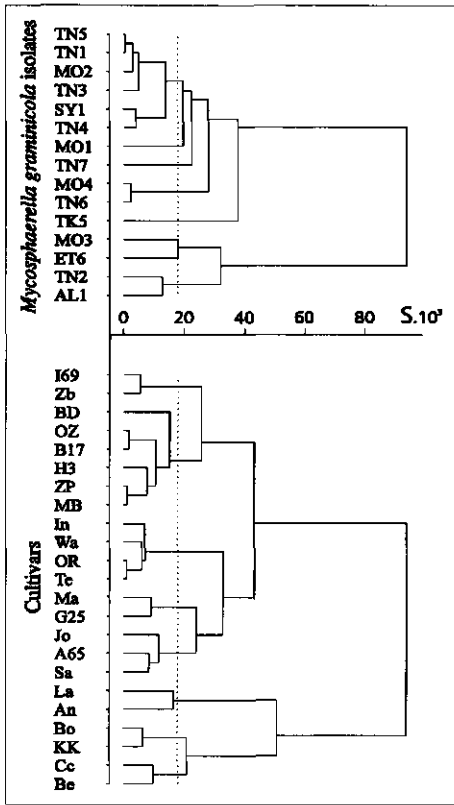
Fig. 3. Dendrograms of simultaneously clustered genotypes of wheat (22) and triticale (1), and *Mycosphaerella graminicola* isolates (15), based on *N* in experiment 2. The positions of the nodes correspond with the cumulative sum of squares for interaction between cultivars and isolates (*S*) on the horizontal axis. The area at the left of the vertical dotted line represents non-significant differences at P=0.05, *S*=18*10³ (for P=0.01, *S*=20*10³).

Three response classes, generally susceptible, generally resistant and differentially resistant, were composed to evaluate the efficacy of the resistance in those cultivars in the different countries, since the number of isolates per country was too small for reliable virulence frequency calculations (Table 9). Apparently, the majority of the bread wheat isolates, except those from Dutch origin, carried virulence for the cvs. Lakhish, Shafir, Gerek 79 and Ceeon. The cvs. Kavkaz, Beth Lehem, Bobwhite, Kavkaz/K4500 1.6.a.4 and Iassul 20, are considered to be appropriate sources for resistance, though their efficacy for Turkey, Kenya, Argentina and The Netherlands may be limited since virulent isolates were found in these countries. Comparison of the responses of cv. Kavkaz and its derivatives 'Kavkaz/K4500 1.6.a.4', 'Kavkaz/7C' and 'Kavkaz/UP301', revealed generally decreased resistance levels in the two latter derivatives, whereas the cross between cvs. Kavkaz and K4500 1.6.a.4, which resulted in the line 'Kavkaz/K4500 1.6.a.4', had a significantly increased level of resistance (Tables 3, 4 and 9). Consideration of the number of cultivars that were generally resistant, isolates originating from Burundi, Rwanda, Uganda and Uruguay, were virulent on fewer cultivars than isolates from other countries (Table 9). However, this may well have coincided with the small number of isolates that originated from these countries, since an increase of isolates would probably result in more cultivars with a differential response. Observed virulences and the origin of the cultivars were not always evident. The resistance in the Dutch cvs. Arminda and Obelisk, for example, was circumvented by several isolates from Turkey, Ethiopia, Uruguay and Argentina, though these cultivars were never exposed to *M. graminicola* outside Europe by commercial cultivation. The efficacy of resistance in the durum wheat cultivars appears to be limited, particularly to the isolates from

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TABLE 6. Adjusted pycnidia (*P*) response matrix of experiment 2; 23 host accessions and 15 *Mycosphaerella graminicola* isolates, arranged according to the clusters of Fig. 4[a]

EC[c]	Cultivars[b]																						
	D Wa	D OR	D Ma	D Te	D ZB	D OZ	D B17	D ZP	D H3	D MB	D I69	D BD	D In	D Cc	D Jo	D A65	D Sa	D G25	B Bo	T Be	B KK	B La	B An
MO1	56	53	53	48	46	51	57	35	58	65	46	61	68	68	62	62	67	20	0	0	0	0	1
TN1	34	40	31	32	61	48	59	29	60	51	28	36	75	63	58	65	62	20	0	0	0	0	0
TN5	14	13	62	69	33	13	19	59	54	48	32	46	76	70	60	67	56	6	-1[d]	-1	-1	-1	-1
MO4	47	48	71	66	45	51	53	54	60	55	33	41	73	58	66	38	39	17	2	2	2	2	2
MO2	42	57	67	67	57	55	69	68	66	56	26	25	74	47	54	46	56	16	-1	-1	-1	-1	-1
TN3	73	70	67	69	72	49	55	54	67	62	33	45	73	50	63	57	60	18	-1	-1	-1	-1	0
TN4	70	61	76	77	63	31	23	69	83	70	24	20	72	59	61	66	48	7	-1	-1	-1	-1	-1
SY1	34	34	63	69	58	58	49	68	67	62	44	38	76	55	59	52	51	12	0	0	0	0	0
TN6	32	16	58	55	53	55	50	60	63	58	40	32	51	48	59	37	45	6	0	0	0	0	0
TN7	47	54	69	64	8	6	6	12	49	57	6	14	62	53	54	32	43	4	2	2	2	2	2
TK5	60	61	34	64	18	5	3	4	5	3	5	4	63	14	24	20	35	5	3	3	3	3	3
TN2	0	13	-2	1	0	-2	-2	-2	-2	-2	-2	-1	10	-2	-1	-2	-2	3	5	-2	0	67	47
ET6	3	21	5	6	1	0	1	1	1	1	0	9	13	0	3	0	8	8	20	0	4	61	54
AL1	15	20	0	1	2	1	1	0	3	1	0	2	39	13	0	4	1	2	21	0	0	61	45
MO3	1	0	3	0	3	2	2	1	0	2	0	0	0	2	0	0	10	3	0	0	0	0	42

[a] $LSD_{0.01}$=17, $LSD_{0.05}$=12.
[a]Experimental codes for cultivars according to Table 1; B: bread wheat, D: durum wheat, T: triticale.
[b]Experimental codes for isolates according to Table 2.
[c]Values <0 are due to block adjustments.

Morocco and Syria, which are extremely virulent. The *T. turgidum* ssp. *dicoccoides* accession G25 is the only entry with a reasonable level of resistance to the majority of the durum wheat derived isolates.

DISCUSSION

GENETIC VARIATION FOR VIRULENCE. Extensive genetic variation for virulence in *M. graminicola*, characterized by differential interactions between host and pathogen genotypes for both *N* and *P* suggests the involvement of specific factors for virulence and resistance in this pathosystem. Specificity in necrotrophic pathogens, such as *Stagonospora nodorum*, *Pyrenophora tritici-repentis*, *Rhynchosporium secalis* (on barley), and *Setosphaeria turcica* (on maize), was reported previously (68,74,105,111), but was considered to be controversial in *M. graminicola*. While some experimental evidence supported its existence (7,31,32,33, 106,138), other evidence did not (88, 134). Hence, Johnson's (56) statement that 'it appears that a gene-for-gene interaction cannot be identified, at least with present techniques'. A major element in this controversy seems to be the limited evidence for differential interactions between host and pathogen genotypes (31,32,33,56,104,138). The most obvious differential interactions reported so far occurred between bread and durum wheat and isolates secured from these species (31,106). An analogous observation was reported by van Ginkel and Scharen (134), who therefore considered specialization in
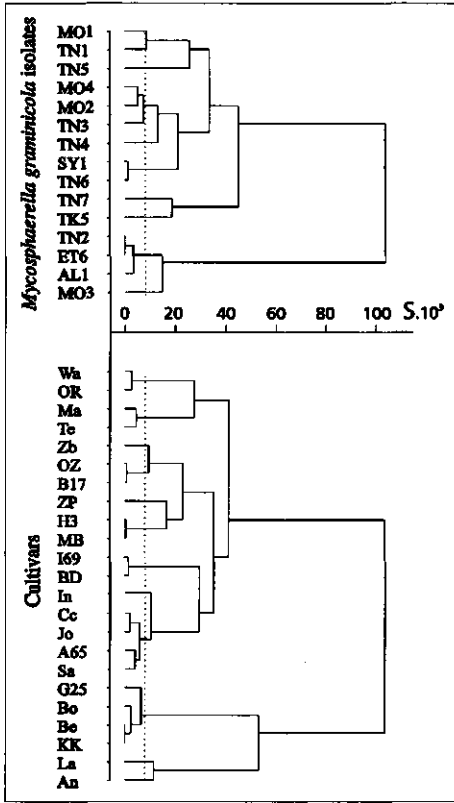
Fig. 4. Dendrograms of simultaneously clustered genotypes of wheat (22) and triticale (1), and *Mycosphaerella graminicola* isolates (15), based on P in experiment 2. The positions of the nodes correspond with the cumulative sum of squares for interaction between cultivars and isolates (S) on the horizontal axis. The area at the left of the vertical dotted line represents non-significant differences at P=0.05, $S=8*10^3$ (for P=0.01, $S=8.5*10^3$).

M. graminicola on bread or durum wheat to be of much greater importance than differential specificity on particular cultivars of these species. Indeed, when considering P in the present study, bread wheat and durum wheat isolates were particularly virulent on bread wheat and durum wheat cultivars, respectively. However, in addition to that, highly significant interactions within each of these systems were determined through analyses of restricted data matrices, either for bread wheat cvs. and bread wheat adapted *M. graminicola* isolates or durum wheat cvs. and durum wheat adapted isolates. This result is in contrast to that of Van Ginkel and Scharen (134), who did not find interactions between host and pathogen genotypes in their experiments, which primarily dealt with durum wheat cultivars. They therefore suggested that interactions may disappear whenever considering a restricted system, *i.e.* bread wheat or durum wheat with their respective isolates. To strengthen the insignificance of isolate x cultivar interaction, they also discussed the relative proportion of the total variance that was attributed to main effects due to cultivars, isolates and interaction, in their own experiments and in the experiments of others (134). Kema et al. (61), however, discussed diverse statistical approaches that were also employed to analyse additional data, and considered it to be incorrect to question the proportion of the MS_{int} as long as it is statistically significant.

THE WIDE genetic variation for virulence in *M. graminicola* complements information on genetic variation revealed by RFLP analyses (10). McDonald and Martinez (81,82) observed a high frequency of RFLPs in a sample of *M. graminicola* isolates, which was mainly secured from one limited area, indicating substantial genetic variation within local populations and even between isolates derived from lesions in the same leaf.

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TABLE 7. Analyses of covariance of the necrosis (*N*) and pycnidia (*P*) disease parameters, on total and restricted response matrices in two experiments[a]

Source of variation	Total response matrix			Restricted response matrix		
		N	P		N	P
	df	MS	MS	*df*	MS	MS
Experiment 1						
isolates	49	5440.5[b]	2587.9[b]	47	4317.2[b]	2872.2[b]
covariates	24	13578.6	2364.4	24	9965.7	2801.6
mainplot error	124	1324.3	331.7	118	1098.7	377.7
cultivars	23	47725.6[b]	38408.1[b]	18	49551.2[b]	35187.5[b]
cultivars * isolates	1127	864.0[bc]	506.4[bd]	846	882.5[be]	542.6[bf]
subplot error	3468	197.9	108.5	2556	182.5	125.0
Experiment 2						
isolates	14	12907.5[b]	20110.0[b]	10	5697.0[b]	5173.2[b]
covariates	9	14054.8	1144.0	9	6811.5	1746.6
mainplot error	36	2687.2	378.7	24	1199.4	513.3
cultivars	22	28722.2[b]	14530.0[b]	17	3549.1[b]	7864.8[b]
cultivars * isolates	308	1220.0[bg]	1347.0[bh]	170	416.5[bi]	730.4[bj]
subplot error	990	191.0	87.1	561	192.1	118.0

[a] The total response matrix of experiment one comprised 24 host accessions and 50 *Mycosphaerella graminicola* isolates, and the restricted response matrix was confined to 19 bread wheat accessions and 48 isolates from bread wheat. The total response matrix of experiment two comprised 23 host accessions and 15 *Mycosphaerella graminicola* isolates, and the restricted response matrix was confined to 18 durum wheat accessions and 11 isolates from durum wheat.
[b] F-value highly significant (P<0.01).
[c-j] Percentages of the total variance are 2.04, 3.58, 2.33, 4.52, 1.24, 1.14, 1.33 and 1.29, respectively.

Indeed, separation of two Kenyan isolates, which originated from the same leaf (*IPO87000-KE1* and *IPO-87011-KE2*), in significantly different clusters indicates the presence of genetic variation for virulence at micro levels. Boeger et al. (10) suggested that common alleles, as defined by probe/restriction enzyme combinations, in very distant *M. graminicola* populations were either due to seed transmission of the pathogen or to the employment of anonymous DNA probes that hybridize to conserved non-coding regions of the genome. In our study, certain cultivars were susceptible to *M. graminicola* isolates that originated from regions where these cultivars were never exposed to the pathogen. Parallel evolution of the pathogen population to imported resistance factors might possibly explain such observations. Since pathogen populations may, at least partly, be structured by gene-for-gene coevolution (124), an integrated analysis of local pathogen populations, using molecular markers and selectable markers such as virulence, would reveal the most useful information for breeding programmes with respect to the magnitude and stability of genetic variation for virulence. In that case, adult plant inoculation experiments should

TABLE 8. Categories of relationships between *Mycosphaerella graminicola* isolates and wheat cultivars[x]

Category	Experiment	Isolate[y]	Response of cultivars[y,z]			
			N		P	
interactive N-	1		Ve	KU	Ve	KU
interactive P		TK8	15[a]	70[b]	1[a]	42[b]
		AR3	68[b]	5[a]	40[b]	-1[a]
	2		Te	MB	Te	MB
		SY1	81[a]	68[a]	69[a]	62[a]
		TK5	67[a]	29[b]	64[a]	3[b]
non-interactive N-	1		T29	Ob	T29	Ob
interactive P		NL3	71[a]	85[a]	4[a]	38[b]
		TK2	85[a]	62[a]	37[b]	3[a]
	2		Wa	Cc	Wa	Cc
		TK5	59[a]	71[a]	60[a]	14[b]
		TN5	68[a]	77[a]	14[b]	70[a]
interactive N-	1		Ve	KZ	Ve	KZ
non-interactive P		AR1	38[b]	5[a]	7[a]	4[a]
		NL3	0[a]	38[b]	-1[a]	2[a]
	2		I69	B17	I69	B17
		TN4	80[a]	77[a]	24[a]	23[a]
		TK5	77[a]	32[b]	5[a]	3[a]
non-interactive N-	1		Ce	Ge	Ce	Ge
non-interactive P		TK4	67[a]	78[a]	59[a]	67[a]
		AR4	62[a]	80[a]	54[a]	68[a]
	2		Te	Ma	Te	Ma
		TN4	83[a]	86[a]	77[a]	76[a]
		MO4	79[a]	99[a]	66[a]	71[a]

[x]Values are taken from Tables 3-6.
[y]Experimental codes, see Tables 1 and 2.
[z]Values followed by different letters are significantly different at P<0.01 (Experiment 1: LSD_N=29 and LSD_P=20; Experiment 2: LSD_N=29 and LSD_P=17).

also be considered since seedling responses not necessarilly correlate with adult plant responses due to the fact that resistance factors may operate in only one of these physiological stages. Adult plant inoculation experiments in the field also showed big cultivar x isolate interactions, and thus confirmed one of the main inferences from the present study that specificity appears to be an important aspect of the wheat - *M. graminicola* pathosystem

TABLE 9. Generalized efficacy of the resistance in bread wheat and durum wheat cultivars, with respect to the pycnidia parameter (*P*), to *Mycosphaerella graminicola* isolates that originated from these respective species[a]

Country and number of isolates	Responses[b] of bread wheat cultivars[c] to bread wheat derived isolates																	
	La	Sh	Ge	Ce	KT	Ol	T29	To	K7	KU	Co	KZ	Ob	Ve	Bo	Ar	BL	KK	Ia
Argentina (6)	●	●	●	●	●	●	o	R	o	o	R	R	o	o	o	o	R	o	o
Uruguay (5)	●	●	●	●	●	●	●	R	R	R	R	R	o	R	o	R	o	R	R
Ethiopia (11)	●	●	●	o	o	o	R	o	o	o	o	R	o	o	o	R	R	o	R
Kenya (8)	●	o	o	●	●	●	o	o	o	o	o	o	o	R	R	R	R	R	R
Burundi (2)	●	●	●	●	●	o	●	●	R	R	o	R	R	o	R	R	R	R	R
Rwanda (1)	●	●	●	●	●	●	●	●	●	●	●	●	●	R	R	R	R	R	R
Uganda (1)	●	●	●	●	●	●	●	●	●	●	●	R	R	R	R	R	R	R	R
Turkey (10)	●	●	●	●	o	o	●	o	o	o	R	o	o	o	R	o	o	R	R
The Netherlands (6)	o	o	o	o	●	o	o	o	o	R	R	o	o	o	o	o	R	R	R

	Responses[b] of durum wheat cultivars[c] to durum wheat derived isolates																	
	Ma	Te	In	Jo	A65	Sa	ZB	OR	Wa	OZ	B17	ZP	I69	BD	Cc	MB	H3	G25
Morocco (3)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	o
Tunisia (6)	●	●	●	●	●	●	o	o	o	o	o	o	o	o	●	●	●	o
Syria (1)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	R
Turkey (1)	●	●	●	●	●	●	●	●	●	●	R	R	R	R	R	R	R	R

[a]Restricted matrices, see foot note a of table 7.
[b]R: no virulent isolates encountered, o: virulent isolates encountered, ●: all isolates carry virulence.
[c]Experimental codes, see Table 1.

(64).

RACES IN *M. GRAMINICOLA*? The occurrence of differential interactions between host and pathogen genotypes suggests a gene-for-gene relationship between these genotypes. However, the pathosystem discussed in the present contribution is far from the ideal gene-for-gene system as discussed by Person (96), which requires a locus in the host that governs either a resistant *or* a susceptible response, and a locus in the pathogen that governs a virulent *or* an avirulent response. Hence, the analysis of data for *M. graminicola*, which have a more quantitative character as compared to

e.g. cereal rusts and powdery mildews, cannot be perfomed sufficiently using the method proposed by Person (96). In the current study, gene-for-gene interaction in the *M. graminicola*-wheat pathosystem was inferred from significant MS_{int} values in ANCOVAs, from clusteranalyses which employed such values as proximity measures for isolates and cultivars in consecutive analyses of variance, and from other statistical procedures (61). Eyal and co-workers (32,33,138) developed an elaborate statistical procedure, which was also adopted by Van Ginkel and Scharen (133,134), to calculate cutpoints in order to assign qualitative descriptors, resistant or susceptible, to

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quantitative data, either N or P. This procedure enabled the designation of hypothetical resistance and virulence genes to host cultivars and pathogen isolates, respectively, and hence analysis of the data as suggested by Person (96). However, the procedure disregards additive modes of action in virulence and resistance, which results in inadequate assignment of hypothetical virulence and resistance factors. Therefore, this procedure was not considered.

THE OCCURRENCE of differential interactions justifies the recognition of physiological races in plant pathology. In case resistance in the host is largely quantitatively inherited, virulence differences among fungal strains might be of insufficient magnitude to distinguish distinct pathogen races. Race-designation would be even more complicated, if not impossible, in case such pathogens have a functional generative stage combined with a relatively efficient dissemination mechanism as compared to the dispersal of asexual propagules, as in *M. graminicola*. Caten (14) argued that extensive genetic variation for virulence, and gene flow between populations of the pathogen, would lead to the designation of a separate race identity to virtually each individual isolate. Indeed, such a situation is conceivable for *M. graminicola*, particularly when considering recombination during ascosporogenesis. Hence, nomenclature of races in *M. graminicola* is fairly trivial. However, the designation of a bread wheat and a durum wheat variant in *M. graminicola* is of importance. Both types can be easily recognized in inoculation experi-

ments, but do not differ morphologically and are not geographically isolated. In addition, the two *M. graminicola* variants could not be distinguished by amplification and digestion of nuclear and mitochondrial internally transcribed spacer ribosomal DNA (ITS rDNA) (E.C.P. Verstappen and G.H.J. Kema, *unpublished*). The sequences of amplified ITS fragments of both variants appeared to be identical (E.C.P. Verstappen, A. Lever, J. Keijer and G.H.J. Kema, *unpublished*) and were also similar to the sequence of *M. graminicola* isolate ATCC#26517 (American Type Culture Collection accession) as was recently published (9), which supports the idea that both variants are from a similar taxonomic rank. A similar situation was recently described for wheat leaf rust, where the durum wheat and bread wheat types clearly differ in pathogenicity and also appear to be sexually isolated, but could not be distinguished by molecular markers (3,34,142). The presence of both *M. graminicola* variants at the same location (e.g. TK5 and TK6 in Altinova-Turkey, and TN1,TN2 and TN3 in Beja-Tunisia) emphasizes the importance of population dynamics studies (10), particularly since *M. graminicola* is of increasing importance in the region.

ALTHOUGH THE major inference of the present study is the specificity of the host-pathogen interaction, which is irrespective of the proposed pathotypes, the suggested gene-for-gene relationship as the underlying mechanism for this requires further evidence through crossing experiments among accessions of host and

pathogen.

GENETIC VARIATION FOR RESISTANCE. The resistance in the host cultivars varied widely in both experiments. In the first experiment, the durum wheat cultivars were in one group, since the majority of the isolates hardly produced pycnidia in them. In contrast, most of the bread wheat cultivars were significantly different for N and P. However, the relationship between some cultivars was evident from composed clusters, such as cv. Kavkaz and its derivatives 'Kavkaz/UP301' and 'Kavkaz/7C'. Another composed cluster contained the cvs. Iassul 20, Bet Lehem and the triticale cv. Beagle, which are apparently unrelated but had low P levels with the majority of the isolates. Cultivars such as 'Kavkaz', 'Bobwhite', 'Kavkaz/K4500 L.A.4' and 'Iassul 20' proved to be highly effective against the majority of the isolates, in accord with Eyal et al. (33).

IN THE second experiment 'OZ 368' and 'Bidi 17' had a similar differential response to the *M. graminicola* isolates, and were clustered. The landrace OZ 368 was selected by Ducellier in 1936 from the landrace population 'Bidi' in the region Oued Zenati, 40 km west of Guelma in Algeria, whereas 'Bidi 17' was selected by Perrot from the same landrace population in 1938 in Guelma (29). A parallel inference is evident for cvs. Hedba 3 and M.B. Bachir, that were selected in 1907 from unknown, but probably similar, landrace populations in the region of Setif, 300 km South-East of Algers (29).

THE RELATION BETWEEN N AND P. Our study considered two disease parameters, N and P, whereas other reports on pathological variation in *M. graminicola* considered either one or the other of these parameters (7,31,32, 33,106,134). The smaller standard error of the mean for P provided a better resolution of genetic variation than N, which was also evident from the cluster analyses. P resulted in more pronounced differences between isolates or cultivars. Therefore, P appears, apart from its epidemiological relevance, to be most appropriate to characterize isolates or cultivars. The controversy about host-pathogen interactions in the wheat - *M. graminicola* pathosystem might be partly due to the analysis of just one parameter, which obviously mitigates the complexity of the pathosystem. Sub - optimal experimental conditions, in particular inadequate relative humidity levels (80,117), may lead to a consideration of N as disease parameter, since pycnidia development will be severely hampered if RH levels are ≤75% or insufficiently controlled. Eyal et al. (33) and Van Ginkel and Scharen (132,133, 134) considered N as their main disease parameter. In addition, the frequency of plants showing pycnidia was determined, sometimes four weeks after inoculation. Yechilevitch et al. (138) reported a mean value of P=3.8 on the resistant cv. Zenati Bouteille, whereas it had a differential response, ranging from P=7 to P=72 with merely durum wheat isolates, in our experiments. Similarly, the susceptible cv. Inbar, had a mean value of P=27.8, whereas in our experiments it ranged from P=50 to P=75 for

various isolates. We observed pycnidium formation already at 10 days after inoculation, and final observations were conducted at 21 days after inoculation. Although these discrepancies may be conferred by differences in virulence of the pathogen isolates, it is not surprising that dissimilar experimental conditions produce conflicting data, thus obscuring the discussion on specificity in this pathosystem (31,32, 33,56,88,134).

OBSERVED CLUSTER discrepancies for N and P suggest that N and P are under different genetical control. Extensive leaf necrosis with no or a few pycnidia occurred frequently. High necrosis levels with varying pycnidial densities were also observed in field experiments (64). Therefore, Rosielle (103)

introduced an assessment scale with six discrete host response classes, representing immunity and varying levels of necrosis and pycnidial density. The reason for dissimilarities between N and P classifications has, to our knowledge, not been addressed. Histological studies showed that phenotypes with high N but low P levels were not profusely colonized (66). These observations suggest that high N levels may be provoked by the pathogen, but apparently could imply avirulence rather than virulence. Therefore, P appears to be the most reliable disease parameter until histological and physiological aspects of the pathogenesis of $M. graminicola$ in compatible and incompatible interactions have been resolved.

Chapter Two

Analysis of Interactions between Pathogen Isolates and Host Cultivars
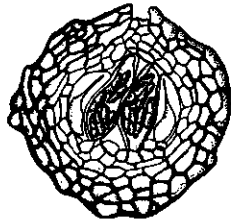
ABSTRACT

Non-parametric and parametric statistical procedures were employed to analyse six data sets comprising 80 pathogen isolates and 47 host cultivars to investigate the presence and relevance of interaction in the wheat - *Mycosphaerella graminicola* pathosystem. Each data set was confined to either responses of bread wheat to bread wheat-derived isolates, or of durum wheat to durum wheat-derived isolates, and to each of two disease parameters presence of necrosis (N) and production of pycndia (P). Four data sets were employed for explorative statistical analyses, which involved a procedure using the size of the overall variances for cultivars and isolates in tables of effects to estimate the relative proportions of specific factors for resistance and virulence in host and pathogen genotypes, respectively. Subsets that comprised cultivars and isolates with either high or low variances, were selected from the data matrices and were subjected to analyses of covariance. Subsets that included entries with high variances revealed interaction mean squares that explained approximately 25% of the total variance, which was considerably higher than in the complete data matrices. The results indicate considerable genetic variation for specific resistance and virulence factors in host and pathogen, respectively, and hence for the effectiveness of the procedure. Analysis of subsets that were confined to entries with low variances resulted in interaction mean squares that contributed little to the total variance, which is an indication for the absence of differential responses, that might due to either susceptible or resistant responses to all applied pathogen isolates. Two data sets were obtained by an additional experiment, involving 15 *M. graminicola* isolates and 24 host cultivars in two replications, which was conducted to design a selection experiment to test hypotheses that were based on preceeding statistical analyses. This experiment, which involved small subsets of isolates and cultivars, confirmed the hypothesis that a large overall variance may be indicative of specific factors for virulence or resistance. It also indicated that a low overall variance is not necessarily indicative of non-specific resistance. In all cases, parametric and non-parametric statistical procedures showed significant interactions between pathogen isolates and host cultivars. Similar results were obtained for both disease parameters, although differences between these parameters are evident. The employed statistical procedures and the additional data demonstrated specificity in the relationship between either bread wheat or durum wheat and *M. graminicola*. This suggests a gene-for-gene relationship in these pathosystems that requires further elucidation and may have important repercussions on breeding strategies.

An ideal gene-for-gene relationship in plant-pathogen interactions requires a locus in the host that governs either a resistant or a susceptible response, and a locus in the pathogen that governs either a virulent or an avirulent response (96). This relationship has often been described using the 'quadratic check' involving two host cultivars and two pathogen isolates, which differ in only one gene for resistance and virulence, respectively. These 'ideal' gene-for-gene relationships are usually confined to pathosystems that involve organisms with genes that confer major effects, which

results in distinct well-defined qualitative disease classes (*e.g.* the cereal rusts). Pathosystems involving host and pathogen genotypes with less pronounced qualitative characteristics are generally studied using quantitative approaches, such as analysis of variance. Gene-for-gene relationships are often suggested by significant statistical interactions between pathogen and host genotypes, but usually without providing unequivocal evidence for specificity in such a pathosystem (32,33,105,111). Thompson and Burdon (124) listed over 40 associations between plants and pathogens that were either demonstrated or suggested to have such an interaction. These examples mostly involved biotrophic pathogens, but included also necrotrophic and hemi-biotrophic pathogens. Others, such as *Mycosphaerella graminicola* (Fuckel) J. Schröt. *in* Cohn (anamorph: *Septoria tritici* Rob. ex Desm.), *Stagonospora nodorum* and *Pyrenophora tritici-repentis*, were not referred to, though a gene-for-gene relationship was also suggested to apply to these pathogens, albeit primarily based on statistical evidence for interaction (33,105,111). Genetic variation for virulence in *M. graminicola*, which is an important fungal pathogen of bread wheat and durum wheat, *Triticum aestivum* L. and *T. turgidum* L. ssp. *durum* L., respectively, has been debated since Eyal et al. (31) suggested physiologic specialization in it. However, the perception of a gene-for-gene relationship in the wheat - *M. graminicola* pathosystem remained controversial. This is primarily due to; 1) the suggestion that specificity for wheat species dominates the specificity

for particular cultivars of those species, 2) the deficiency of examples of cultivars that quickly succumbed to new strains of the pathogen, and 3) the small proportions of the total variance in analyses of variance that were explained by interaction (32,33,56,134). In addition, Van Ginkel and Scharen (133) performed a diallel analysis, and found general combining ability (GCA) to be the major component of variation, though specific combining ability (SCA) effects were also significant. A generation mean analysis also indicated the importance of additive gene effects (132). They, therefore, suggested that their results could indicate the absence of gene-for-gene relationships, implying genetic variation for aggressiveness rather than for virulence among *M. graminicola* isolates (134,133).

Leonard (74) reviewed research on northern leaf blight of maize caused by *Setosphaeria turcica*. In this pathosystem it was also demonstrated that GCA and SCA effects were significant, though GCA effects were much larger than SCA effects, and a generation mean analysis indicated that additive gene action was of major importance. Nevertheless, there was also evidence for specificity, though field populations did not provoke a detectable decline of quantitative resistance. In this pathosystem, and in the relation between *Cochliobolus heterostrophus* and maize, there is conclusive evidence of adaptation by the pathogen to (partially) overcome polygenic resistance in maize (74). However, the genetic differentiation among *C. heterostrophus* strains was not always apparent from the interaction mean squares (MS_{int}) in

analyses of variance, which were therefore not considered to be entirely appropriate to demonstrate specificity in a pathosystem. Significance of host x pathogen interactions may depend on experimental design, actual proportions of genes with general and specific effects (52,54,74,75) or experimental conditions (28,53). Moreover, Vanderplank (129) pointed out that parametric analyses, including analysis of variance, are sensitive to the way disease is assessed, a problem which is circumvented by the application of non-parametric procedures.

Kema et al. (61) discussed genetic variation for virulence and resistance in the wheat - *M. graminicola* pathosystem, and considered two disease parameters *i.e.* the presence of necrosis and pycnidia. Highly significant MS_{int} values for each of these parameters were determined in analyses of covariance (ANCOVAs), which involved separate analyses of data sets from either bread wheat or durum wheat, and *M. graminicola* isolates originating from these species.

In the present study parametric and non-parametric statistical analyses are employed to 1) test for specificity in the wheat-*M. graminicola* pathosystem, and 2) to select cultivars and isolates with presumed specific factors for resistance and virulence, respectively, in order to investigate the reproducibility of observed interactions between such cultivars and isolates.

MATERIAL AND METHODS

NON-PARAMETRIC AND EXPLORATIVE PARAMETRIC STATISTICAL ANALYSES. Four data sets, which were adjusted

for block *i.e.* time effects (61), were available for further statistical analyses. They comprised 19 bread wheat cultivars and 48 bread wheat derived isolates and 18 durum wheat cultivars and 11 durum wheat derived isolates. The two disease parameters, N for necrosis and P for pycnidia estimated as percentages on the primary leaves, respectively (61), were studied individually.

The non-parametric method involved a procedure for two-way layouts, according to De Kroon and Van der Laan (23). These authors introduced a concept of rank-interaction between the classifying factors of a two-way table with equal numbers of observations per cell, and a concomitant distribution-free test for significance. Essentially, the procedure involves a combination of the Kruskal-Wallis and Friedman non-parametric tests. For large samples the test statistics of both tests are approximately chi-square distributed. Kruskal-Wallis test statistics are separately calculated for differences between cultivars (treatments) by assigning rank numbers to the samples *within* each isolate (block), and are subsequently cumulated *over* blocks. The Friedman statistic for differences between cultivars is calculated for the complete table, classified by isolates and cultivars. The cumulated Kruskal-Wallis statistic T is decomposed into two components, T_1 and $T_2 = T - T_1$, where T_1 is the aforementioned Friedman statistic. Under H_0, the test statistics T_1 and T_2 are approximately independent and distributed according to a chi-square distribution. Statistic T_1 accounts for differences between cultivars, whereas

T_2 is sensitive to differences in ranking orders of host cultivars within isolates. A suggested procedure to correct for main effects by calculating the sample medians for each row and column to calibrate the data was adopted (23), and the equality of the ranking orders of the host cultivars for the *M. graminicola* isolates was tested.

The explorative statistical procedure involved parametric analyses as proposed by Eberhart and Russell (27). They employed regression analysis for estimating stability parameters to compare cultivars over different environments, which was adapted by Leonard and Moll (75) for host-pathogen systems. The model was further advanced by Jenns et al. (52), and later simplified by Jenns and Leonard (54). The procedure is of particular interest, since it provides estimates for relative specificity in pathosystems involving quantitative aspects of resistance and/or virulence, and was therefore considered to be appropriate for a further evaluation of specificity in the wheat-*M. graminicola* pathosystem (61). Hence, the data sets were subjected to analyses of covariance (ANCOVAs), and a table of effects was generated for each data set, which comprised only interaction components for each host-isolate combination, *i.e.* actual disease severities minus main effects for cultivars and isolates. This table of effects was used to calculate overall variances for both disease parameters (σ^2_N and σ^2_P) for each isolate and cultivar to estimate the relative specificity for virulence and resistance in pathogen and host genotypes, respectively (54).

ANCOVAs on subsets of isolates and cultivars were employed to study the effects of matrix size and selection on the proportion of the MS_{int} in the total variance.

ADDITIONAL EXPERIMENTS. To substantiate the evidence for specificity in the wheat - *M. graminicola* pathosystem and to evaluate the aforementioned suggested statistical procedures empirically, two additional experiments were conducted. The first experiment involved inoculations of a tester set of 24 accessions, that was similar to the one employed in previous experiments (61), with 15 monopycnidial *M. graminicola* isolates, that were obtained according to procedures described earlier (60), and were stored over silicagel at -20 C and at -80 C before use (Table 1). The second experiment was conducted after selection of cultivars and isolates with high σ^2_P values and comprised four bread wheat cultivars and three bread wheat adapted *M. graminicola* isolates, and two durum wheat cultivars and two durum wheat adapted *M. graminicola* isolates.

EXPERIMENTAL DESIGN AND DATA ANALYSES. The first experiment was carried out according to a split-plot design with two replications and was analysed by analysis of variance. The experimental procedures and conditions were similar to those described earlier (61). The data enabled the selection of isolates and cultivars, which were suggested to carry a relatively large fraction of specific factors (high σ^2_P values). Four years later these isolates and cultivars were again tested in a split-plot verification

TABLE 1. Experimental code and origin of 15 *Mycosphaerella graminicola* isolates that were studied for genetic variation for virulence towards 23 wheat cultivars and one triticale cultivar.

ECI[a]	Isolate	Country	Location
MX1	IPO90001	Mexico	Toluca
MX2	IPO90002	Mexico	Toluca
MX3	IPO90003	Mexico	Toluca
MX4	IPO90004	Mexico	Patzcuaro
MX5	IPO90005	Mexico	Juchitepec
MX6[b]	IPO90006	Mexico	Toluca
MX7	IPO90007	Mexico	Toluca
MX8	IPO90008	Mexico	Juchitepec
MX9	IPO90009	Mexico	Juchitepec
MX10	IPO90010	Mexico	Juchitepec
MX11	IPO90011	Mexico	Juchitepec
MX12[b]	IPO90012	Mexico	Patzcuaro
EC1	IPO90013	Ecuador	Alausi
PU1	IPO90014	Peru	Andenes
PU2[b]	IPO90015	Peru	Andenes

[a]Experimental codes for isolates.
[b]Isolates selected for specificity tests.

experiment with two replications over time. An interaction between two durum wheat cultivars and two durum wheat-adapted *M. graminicola* isolates was retested in the same experiment. The statistical analysis of the verification experiment differed from the analyses of the large previous experiments (see also 61), since the degrees of freedom for the residual variance are coherent with the size of experiments. Transformation of the data in large experiments (61) did not substantially stabilize the residual variance and did not influence the conclusions. In small experiments, however, stabilization of the residual variance is appropriate. The verification experiment was, therefore, analysed by using a generalized linear model (GLM) with logit link and variance function proportional to $M(100-M)$, where M is the mean disease parameter.

RESULTS

NON-PARAMETRIC STATISTICAL ANALYSES OF BREAD WHEAT AND DURUM WHEAT DATA SETS. The distribution-free procedure for two-way layouts showed the presence of rank-interactions since the T_2 test statistics were significant at $P<0.01$ (Table 2). Exposing cultivars to different *M. graminicola* isolates in both systems, and for both response parameters, resulted in significant ranking differences. The non-significance of the T_1 statistic was due to the correction for main effects (Table 2).

PARAMETRIC STATISTICAL ANALYSES OF BREAD WHEAT AND DURUM WHEAT DATA SETS. Calculation of σ^2_P for each cultivar involved, and subsequent ranking from low to high provides, together with the overall means, a useful estimator of relative specificity of the resistance in these cultivars (Tables 3 and 4). A high σ^2_P level is always an indication for fluctuating responses toward *M. graminicola* isolates, hence possibly for a relatively large proportion of specific resistance factors. Low σ^2_P levels, however, suggest either, a relatively large proportion of general resistance factors, or a relatively small proportion of such factors, *i.e.* no or unmatched specific resistance. Cultivars such as 'Veranopolis' and 'Kavkaz/7C', had among the highest σ^2_P, but low overall P levels, and are therefore considered highly differential in their response to

TABLE 2. Non-parametric analysis for two-way layouts, involving response matrices for the bread wheat-*Mycosphaerella graminicola* pathosystem (48 isolates and 19 cultivars), and the durum wheat-*Mycosphaerella graminicola* pathosystem (11 isolates and 18 cultivars), and the disease parameters N (necrosis) and P (pycnidia) estimated as percentages on primary wheat leaves, averaged over pots (see chapter 1).

Patho-system	T^a	df^b	T_1^c	df	T_2^d	df
BW^e						
N	1839^f	864	15^g	18	1824^f	846
P	1908^f	864	13^g	18	1895^f	846
DW^e						
N	253^f	187	10^g	17	243^f	170
P	425^f	187	5^g	17	420^f	170

[a]T=Kruskal-Wallis statistic, for differences between cultivars, cumulative for the number of blocks, *i.e.* isolates, involved.
[b]df=degrees of freedom, calculated as I(C-1) for T, C-1 for T_1, and (C-1)(I-1) for T_2, where I is the number of isolates and C the number of cultivars involved.
[c]T_1=Friedman statistic, for differences between blocks, *i.e.* isolates.
[d]T_2=Statistic for rank interaction.
[e]*BW*=bread wheat, *DW*=durum wheat.
[f]Highly significant, P<0.01, χ^2_{df} approximation.
[g]Not significant, due to the correction for cultivar main effects.

M. graminicola, whereas cultivars like 'Iassul 20', 'Beth Lehem', 'Kavkaz/K4500 1.6.a.4' and 'Bobwhite' respond more or less similarly to most of the isolates (low P and low σ^2_P). Similar inferences hold for the durum wheats and wild emmer accession *T. dicoccoides* G25 (Table 4). 'G25', having a low σ^2_P, could carry a large proportion of factors for general resistance or unmatched specific resistance factors

compared to cv. Om Rabi 5, whereas cvs. such as 'Jori', with a low σ^2_P and a high overall P, are expected to carry small proportions of general resistance factors and effective specific resistance factors.

Evidently, considerations for N and P were not congruent, as exemplified by the bread wheat cvs. Arminda and Kavkaz which had among the highest σ^2_N values, suggesting a relatively large proportion of specific resistance factors, whereas the σ^2_P values directed more towards a relatively small proportion of such factors (Table 3). In contrast, the durum wheat cultivars had almost invariably high N levels, thus relatively low σ^2_N values were expected (Table 4).

An analogous procedure for the *M. graminicola* isolates provides estimations for the relative proportion of specific virulence factors in these isolates (Tables 5 and 6). The σ^2_P for bread wheat and durum wheat derived isolates ranged from about 40-400. Isolate *IPO89013* had a high σ^2_P but a low overall P, hence carries specific virulence for only a few cultivars. Other isolates with comparable magnitudes of σ^2_P showed a much higher overall P level, and could therefore carry a larger number of specific virulence factors. Again comparisons between σ^2_P and σ^2_N were largely contrasting and lead to contradictory hypotheses for the proportions of specific and general virulence factors.

To investigate the effect of matrix size and selection on the proportion of the MS_{int} in the total variance, several ANCOVAs were conducted on restricted data sets. Random restriction of the data matrices, for both N and P,

TABLE 3. Estimates for relative specificity of resistance in 19 bread wheat cultivars to 48 *Mycosphaerella graminicola* isolates, quantified and ordered by the size of the calculated overall variance for the disease parameters P and N, σ^2_P and σ^2_N, respectively, for each cultivar in the tables of effects. Figures are based on four replicates over time (see chapter 1).

Cultivar	ECC[a]	σ^2_P	$P_{overall}$[b]	σ^2_N	$N_{overall}$[b]
Iassul 20	Ia	49	5	160	46
Beth Lehem	BL	52	6	78	38
KZ/K4500	KK	74	5	229	25
Bobwhite	Bo	79	8	101	25
Ceeon	Ce	90	34	133	51
Kavkaz	KZ	95	10	480	48
Arminda	Ar	96	6	411	38
KZ/UP301	KU	103	11	210	28
Colotana	Co	105	11	112	50
Lakhish	La	115	37	109	57
Shafir	Sh	118	40	112	63
Toropi	To	153	18	206	62
Taichung 29	T29	162	32	75	77
Gerek 79	Ge	172	41	190	73
Olaf	Ol	181	28	232	50
Obelisk	Ob	182	24	163	58
Veranopolis	Ve	202	13	505	32
Klein Titan	KT	222	42	146	76
Kavkaz/7C	K7	225	17	412	44

[a]Experimental codes for cultivars.
[b]Mean over 48 *M. graminicola* isolates, in four replications, for each cultivar.

TABLE 4. Estimates for relative specificity of resistance in 18 durum wheat cultivars to 11 *Mycosphaerella graminicola* isolates, quantified by calculation of the overall variance for the disease parameters P and N, σ^2_P and σ^2_N, respectively, for each cultivar in the tables of effects. Figures are based on four replicates over time (see chapter 1).

Cultivar	ECC[a]	σ^2_P	$P_{overall}$[b]	σ^2_N	$N_{overall}$[b]
Jori	Jo	36	56	47	66
G25	G25	57	12	96	55
Safir	Sa	70	51	111	64
Inrat 69	I69	80	29	116	79
Inbar	In	86	69	77	73
M.B. Bachir	MB	117	53	93	72
Cocorit	Cc	124	53	71	75
Acsad 65	A65	127	49	159	60
Hedba 3	H3	132	57	156	82
BD 2777	BD	140	33	133	81
Z. Bouteille	ZB	160	47	114	85
Marzak	Ma	167	59	82	80
OZ 368	OZ	218	38	102	73
Tensift	Te	243	61	41	81
ZB/TP[c]	ZP	276	46	45	75
Bidi 17	B17	287	40	122	68
Waha	Wa	368	46	94	63
Om Rabi 5	OR	418	46	109	57

[a]Experimental codes for cultivars.
[b]Mean over 11 *M. graminicola* isolates, in four replications, for each cultivar.
[c]*T. polonicum.*

resulted in larger proportions of the MS_{int}, which suggests that the size of a data matrix, irrespective of the genotypes involved, influences the proportion of the MS_{int} (not shown). Selected small subsets for N and P that comprised either five bread wheat or durum wheat cultivars, and five isolates from these species with either the highest or the lowest σ^2_P levels (Tables 3 and 4), were analyzed as described by Kema et al. (61). The subsets including cultivars and isolates with high σ^2_P levels resulted in MS_{int}

proportions of over 25%, whereas analysis of subsets which included cultivars and isolates with low σ^2_P levels, determined MS_{int} proportions of approximately 1% (Table 7). Similar results were obtained for N (not shown). Since significance tests could not be performed due to the *a posteriori* approach of this analysis, additional experiments were performed.

PARAMETRIC AND NON-PARAMETRIC STATISTICAL ANALYSES OF TWO ADDITIONAL EXPERIMENTS. The responses of the

TABLE 5. Estimates for relative specificity of virulence in 48 *Mycosphaerella graminicola* isolates to 19 bread wheat cultivars, quantified by calculation of the total variance for the disease parameters P and N, σ^2_P and σ^2_N, respectively, for each isolate in the table of effects. Figures are based on four replicates over time (see chapter one).

Isolate	σ^2_P	$P_{overall}$[a]	σ^2_N	$N_{overall}$[a]
IPO87018	39	17	100	46
IPO88027	43	12	146	34
IPO88014	44	16	51	57
IPO87000	45	13	127	55
IPO87008	51	16	110	48
IPO88024	55	15	169	57
IPO88010	57	28	91	61
IPO88021	60	20	193	62
IPO87013	61	16	105	52
IPO87011	69	25	101	58
IPO86063	71	18	216	49
IPO86026	73	20	103	58
IPO86078	74	15	333	44
IPO86010	78	24	107	52
IPO88023	80	22	87	49
IPO87015	81	19	121	45
IPO87012	81	15	199	50
IPO87021	89	23	275	45
IPO88018	101	24	196	48
IPO235	105	7	320	44
IPO88005	109	25	123	66
IPO88019	111	23	176	58
IPO86013	111	29	143	51
IPO87009	114	20	132	47
IPO87020	123	15	196	50
IPO88020	125	22	103	46
IPO89012	131	6	258	35
IPO88025	131	6	318	42
IPO88038	131	21	145	53
IPO89010	133	16	286	59
IPO87019	139	16	290	41
IPO86008	145	22	289	41
IPO86023	150	23	209	42
IPO87016	150	24	173	42
IPO86068	160	33	128	56
IPO87024	169	23	342	60
IPO88037	169	25	314	54
IPO88013	176	13	271	33
IPO87023	179	29	298	45
IPO88015	180	27	161	51
IPO88016	199	32	183	55
IPO86009	227	26	209	51
IPO88004	238	22	418	50
IPO89013	243	8	776	31
IPO87022	272	26	481	45
IPO89011	327	30	296	57
IPO88017	356	21	403	49
IPO88022	402	24	346	46

[a]Mean over 19 bread wheat cultivars, in four replications, for each isolate.

TABLE 6. Estimates for relative specificity of virulence in 11 *Mycosphaerella graminicola* isolates to 18 durum wheat cultivars, quantified by calculation of the total variance for the disease parameters P and N, σ^2_P and σ^2_N, respectively, for each isolate in the table of effects. Figures are based on four replicates over time (see chapter 1).

Isolate	σ^2_P	$P_{overall}$[a]	σ^2_N	$N_{overall}$[a]
IPO91020	46	52	129	77
IPO91011	60	53	37	80
IPO91016	79	56	81	78
IPO91018	94	52	36	66
IPO91017	117	53	115	83
IPO91015	146	37	141	58
IPO91012	176	43	66	76
IPO91009	203	47	41	78
IPO91004	245	52	115	70
IPO91014	263	46	18	70
IPO86022	399	25	260	52

[a]Mean over 18 durum wheat cultivars, in four replications, for each isolate

durum wheat and triticale cultivars in the first additional experiment were not included in the tables (Tables 8 and 9) since the *M. graminicola* isolates were adapted to bread wheat, particularly for P, which substantiates the evidence for a bread wheat and a durum wheat variant in *M. graminicola* (61).

Cluster analyses similar to those described by Kema et al. (61) grouped the 15 *M. graminicola* isolates (Table 1) in four and three significantly different clusters for P and N, and the cultivars in five and three significantly different clusters for P and N, respectively (not shown). Hence, cultivars or isolates that were clustered for N, not necessarily constituted a similar cluster for P, and *vice versa*, which suggests that both parameters are under different genetic control, as was suggested previously (61). For P the isolates were largely separated by

TABLE 7. Analyses of covariance of the disease parameter P on four data subsets, including five host and pathogen genotypes, which were confined to either bread or durum wheat, and *Mycosphaerella graminicola* isolates which were adapted to these host species. Each subset included entries with either high or low σ^2_p levels, suggesting a relatively large and small proportion of specific factors for resistance and virulence, respectively.

	Bread wheat		Durum wheat	
Source of variation	df	MS	df	MS
High σ^2_p levels				
isolates	4	2342	4	1269[a]
covariates	13	471	9	257
mainplot error	1	16	6	75
cultivars	4	2072	4	4241
cultivars * isolates	16	1760[a]	16	1991[b]
subplot error	55(1)	95	60	119
Low σ^2_p levels				
isolates	4	17	4	169
covariates	13	35	9	670
mainplot error	1	22	6	200
cultivars	4	2105	4	9705
cultivars * isolates	16	39[c]	16	166[d]
subplot error	69(1)	37	60	133

[a-d]Percentages of the total variance are 26, 25, 1 and 2, respectively.

from Patzcuaro (*IPO90004-MX4* and *IPO90012-MX12*).

Analyses of variance and the non-parametric procedure of De Kroon and van der Laan (23) indicated significant cultivar x isolate interactions (Tables 10 and 11). In order to enable the selection of isolates and cultivars with supposedly high proportions of specific virulence and resistance factors, respectively, the data were analyzed according to the afore-mentioned procedure (54), and the isolates and cultivars were arranged according to the size of the calculated variances (σ^2_N and σ^2_P, Tables 8 and 9). Hence, isolates and cultivars with assumed specific factors for virulence and resistance, respectively, appeared in the right-bottom part of the tables. Selection of an appropriate subset of cultivars and isolates for the second additional experiment therefore considered that section of these tables. Cultivars Olaf, Veranopolis and Kavkaz were selected and supplemented by cv. Kavkaz/K4500 1.6.a.4 for a specificity test. The selected isolates *IPO90015-PU2*, *IPO90012-MX12* and *IPO90006-MX6*, seemed to carry specific virulence factors for resistance factors in several of these cultivars, including cv. Kavkaz/K4500 1.6.a.4 (Tables 8 and 9). Isolates *IPO86022-TK5* and *IPO91014-TN6* and cvs. Waha and Hedba 3 were selected to retest an interaction that was observed in a previous experiment (61) between these durum wheat cultivars and durum wheat adapted *M. graminicola* isolates.

The results of this second additional experiment (Table 12) confirmed previous data (Table 9, see also Table 6 in

location, except for the isolates from Toluca and the two isolates from Peru. Isolates *IPO90001-MX1*, *IPO90006-MX6* and *IPO90007-MX7* from Toluca were separated from the others due to the combined virulence for cvs. Olaf and Kavkaz and its derivatives. The other isolates from Toluca (*IPO90002-MX2* and *IPO90003-MX3*) were virulent on cv. Olaf but not on cv. Kavkaz and its derivatives, whereas the reverse was observed for the isolates

TABLE 8. Necrosis (*N*) response matrix of 19 bread wheat accessions to 15 *Mycosphaerella graminicola* isolates. Values are means of two replicates. Isolates and cultivars are arranged according to the size of their variance (σ^2_N) in the table of effects. $LSD_{0.01}=53$, $LSD_{0.05}=40$.

EC[c]	Cultivars[b]																			σ^2_N
	Bo	KT	Ob	BL	Ia	La	Sh	T29	Co	To	Ce	Ge	KK	Ar	KU	Ol	Ve	K7	KZ	
MX11	18	72	55	25	48	34	48	77	41	40	21	37	3	29	3	21	9	10	18	151
PU1	7	53	35	8	53	33	13	55	11	61	20	28	6	11	5	21	53	3	3	172
MX5	20	82	73	40	92	76	66	78	39	66	49	58	8	58	34	72	37	39	20	185
MX9	19	83	49	9	69	55	39	72	31	45	5	26	5	41	13	46	25	10	13	193
MX10	20	94	81	44	96	46	80	99	40	51	30	57	18	31	30	60	44	39	28	196
MX3	9	81	39	16	56	50	37	88	53	65	51	75	4	5	5	60	30	12	9	201
MX2	34	91	61	27	72	73	49	100	65	79	80	90	15	14	25	86	50	49	24	229
EC1	19	93	53	14	85	56	61	90	54	92	59	95	4	28	23	73	52	26	20	233
PU2	4	60	39	5	50	63	43	85	24	74	56	53	3	28	6	48	66	3	8	304
MX8	5	73	69	23	69	57	49	99	19	43	46	82	7	53	5	52	68	10	8	369
MX7	36	75	75	21	87	63	61	98	72	93	54	87	32	10	82	82	25	97	92	405
MX1	42	94	75	40	73	69	61	92	74	93	29	44	72	22	76	53	34	95	87	434
MX6	30	91	61	41	66	87	55	88	44	73	41	75	73	30	82	66	8	100	82	469
MX12	38	66	45	28	69	28	31	53	27	58	19	79	33	6	3	7	9	10	85	498
MX4	41	93	90	35	93	66	50	88	54	81	68	99	74	51	81	5	42	99	96	552
σ^2_N	89	101	106	118	131	142	154	156	157	168	302	359	381	396	441	584	597	740	778	

[a,b]Experimental codes for isolates and cultivars, respectively.

TABLE 9. Pycnidia (*P*) response matrix of 19 bread wheat accessions to 15 *Mycosphaerella graminicola* isolates. Values are means of two replicates. Isolates and cultivars are arranged according to the size of their variance (σ^2_P) in the table of effects. $LSD_{0.01}=32$, $LSD_{0.05}=24$.

EC[c]	Cultivars[b]																			σ^2_P
	BL	Co	Bo	KK	KT	To	Sh	Ar	Ia	La	Ce	Ge	Ob	T29	KU	Ol	KZ	Ve	K7	
MX8	2	1	1	0	30	2	32	9	7	20	25	34	40	56	0	29	0	0	5	45
MX11	0	3	3	0	19	2	40	1	4	9	9	15	39	47	0	7	1	0	0	69
MX5	9	11	9	2	29	16	51	20	18	49	33	41	43	70	6	48	3	3	7	87
MX3	2	4	0	0	26	19	41	0	0	31	40	44	24	74	0	39	0	12	0	91
MX10	3	4	7	5	38	8	41	11	9	29	19	24	62	71	8	32	0	2	9	95
EC1	0	2	12	0	36	25	52	3	5	16	33	45	17	72	4	44	1	5	7	103
MX9	1	7	7	0	29	7	45	2	12	49	3	11	34	49	0	39	0	0	5	111
MX7	2	20	31	13	26	31	43	0	1	28	42	56	41	64	43	50	41	0	38	128
PU1	0	0	0	0	4	7	8	1	1	18	15	5	8	30	0	6	0	20	0	143
PU2	0	1	0	0	30	19	37	0	5	25	37	32	17	58	0	28	0	43	0	148
MX12	1	1	18	3	25	27	30	0	0	14	12	49	33	40	25	0	32	0	40	171
MX2	3	28	20	2	45	48	32	1	3	55	57	60	42	73	4	59	0	1	24	201
MX6	19	13	21	30	56	40	61	4	1	67	33	53	45	73	49	46	58	0	63	211
MX4	3	4	10	13	17	19	42	8	3	39	42	49	59	25	20	0	15	0	45	212
MX1	16	24	29	36	54	35	53	1	3	52	23	30	47	65	55	33	58	5	66	265
σ^2_P	28	35	35	47	65	68	82	105	111	147	161	167	173	181	186	243	266	274	303	

[a,b]Experimental codes for isolates and cultivars, respectively.

TABLE 10. Analyses of variance of the disease parameters N and P of response matrices (Tables 8 and 9, respectively) that comprised 19 bread wheat cultivars and 15 *Mycosphaerella graminicola* isolates, originating from Mexico, Peru and Ecuador.

		N		P
Source of variation	df	MS	df	MS
isolates	14	6920[a]	14	2974[b]
mainplot error	14	2363	14	75
cultivars	18	10738[b]	18	7052[b]
cultivars * isolates	252	656[b,c]	252	297[b,c]
subplot error	270	316	270	130

[a,b]F-values significant at P<0.05 and P<0.01, respectively.
[c]The percentage of the total variance for both N and P is 3.

TABLE 11. Non-parametric analysis for two-way layouts, involving the disease parameters N and P of response matrices that comprised 19 bread wheat cultivars and 15 *Mycosphaerella graminicola* isolates, originating from Mexico, Peru and Ecuador (Tables 8 and 9, respectively).

Disease parameter	T[a]	df[b]	T_1[c]	df	T_2[d]	df
N	311[e]	270	7[g]	18	304[e]	252
P	346[f]	270	7[g]	18	339[f]	252

[a]Kruskal-Wallis statistic, for differences between cultivars, cumulative for the number of blocks, *i.e.* isolates, involved.
[b]df=degrees of freedom, calculated as I(C-1) for T, C-1 for T_1, and (C-1)(I-1) for T_2, where I is the number of isolates and C the number of cultivars involved.
[c]Friedman statistic, for differences between blocks, *i.e.* isolates.
[d]Statistic for rank interaction.
[e]Significant at P<0.05, χ^2_{df} approximation.
[f]Highly significant, P<0.01, χ^2_{df} approximation.
[g]Not significant.

61) with regard to specificity, hence demonstrated once more interactions between host cultivars and pathogen isolates in both pathosystems. For the bread wheat isolates the P level of some cultivars was considerably higher than in the first additional experiment, but did not influence the conclusions on interaction. The cultivar x isolate component of the accumulated analysis of deviance for N (not shown) and P was highly significant for both pathosystems (Table 13).

DISCUSSION

STATISTICAL INTERACTION is regularly suggested to be an indicator for specificity of virulence and resistance in host-pathogen systems (32,33,105, 111). However, lack of statistical interaction not necessarily supports the absence of specificity since interaction effects might be so small that they will not be detected (95), or might be dependent on environmental conditions (28,53, 140). Essentially, the biological implication of statistical evidence for interaction would be strengthened by providing unequivocal examples of interactions between certain host and pathogen genotypes (61). In addition non-parametric statistics for interaction would strengthen hypotheses of gene-for-gene relationships between such genotypes, since such analyses avoid problems of stretch, *i.e.* non-homogeneity of the error.

IN THE present study of the bread and durum wheat pathosystems, the ranking of cultivars differed significantly between isolates for both response parameters, which corroborates previous data and suggestions for specificity in these pathosystems. Additio-

TABLE 12. Pycnidia (*P*) response matrix of four bread wheat accessions to three bread wheat adapted *Mycosphaerella graminicola* isolates, and of two durum wheat cultivars to two durum wheat adapted *Mycosphaerella graminicola* isolates.

Isolates	Bread wheat cultivars[a]			
	KK	KZ	Ve	Ol
IPO90006 (MX6)	46[b,c]	60[c]	5[d]	70[c]
IPO90012 (MX12)	46[c]	69[c]	8[d]	4[d]
PU2	5[d]	6[d]	66[c]	70[c]

	Durum wheat cultivars[a]	
	Wa	H3
IPO91014 (TN6)	13[f]	57[e]
IPO86022 (TK5)	51[e]	7[f]

[a]Experimental codes for cultivars.
[b]Values are backtransformed means of data analyzed on logit scale, from two experiments (bread wheat cultivars vs. bread wheat derived isolates, and durum wheat cultivars vs. durum wheat derived isolates) that were conducted over time and comprised two and three replications, respectively. Pairwise comparisons in each experiment were performed between all cells by approximate t-tests on the transformed scale. Significant differences are indicated by different letters (c-d, and e-f).

TABLE 13. Accumulated analyses of deviance of the disease parameter *P* in two verification experiments (Table 12) that comprised four bread wheat cultivars and three bread wheat adapted *Mycosphaerella graminicola* isolates, and two durum wheat cultivars and two durum wheat adapted *Mycosphaerella graminicola* isolates, respectively.

	Bread wheat system		Durum wheat system	
Change	df	MD[a]	df	MD[a]
blocks	1	113[c]	2	33[b]
cultivars	3	28[b]	1	0[b]
isolates	2	17[b]	1	6[b]
cultivars * isolates	6	150[c]	1	317[c]
Error	11	8	6	8

[a]MD=Mean deviance.
[b,c]F-values not significant and significant at P<0.01, respectively.

nal analyses involved ANCOVAs of subsets, that contained selected wheat cultivars and *M. graminicola* isolates with either low or high σ^2_P values, which were, according to a theoretical model system (54), considered to carry relatively small and large proportions of specific factors, respectively. Analysis of data sets including host and pathogen genotypes, that were considered to carry relatively small proportions of specific factors, revealed only a small proportion of the total variance that was explained by interaction, which was of similar size as those described to be significant in other reports (33,55,95,111). The large proportion of the total variance that was explained by interaction in the data set with high σ^2_P values is not inevitable, since high σ^2_P levels do not provide any insight in the genetic structure of the entries. Two entries may have high σ^2_P values and a similar genetic background, hence the variance will be explained by main effects. Whenever the interaction component explains a significant proportion of the total variance, different specific resistance and virulence factors may be hypothesized. Therefore, the result supported the hypotheses that the genotypes involved carry different specific factors for resistance and virulence, and that the proportion of the MS_{int} depends on matrix size and

on unknown proportions of specific factors in the genotypes involved, which is in accordance with previous data and theoretical models (54,61,95). In addition, these hypotheses were also strengthened in two additional experiments, which substantiated the evidence for specificity in the wheat-*M. graminicola* pathosystem. It is an actualization of Ellingboe's (28) statement that nonspecific resistance should be interpreted as resistance that has not yet shown to be specific. Resolving specificity, therefore, seems to depend on many factors, among which optimal experimental conditions may be of prime importance (32,53,61,140).

JENNS AND Leonard's procedure (54) to order host and pathogen genotypes according to σ^2_P, which is suggested to be correlated with the proportion of operational specific factors, pointed to cultivars with high σ^2_P values that might be employed as 'differentials' to elucidate the virulence structure in *M. graminicola* populations. However, cultivars with a low σ^2_P and a low overall *P* level do not necessarily carry relatively large proportions of factors for general resistance, and thus could not be classified as potentially durable. Even when dealing with a pathosystem involving typically quantitative aspects of resistance and virulence, such a prediction would be misleading. This warning is illustrated by the virulence for the resistance in cv. Kavkaz/K4500 1.6.a.4, which was found in isolates *IPO90006*-MX6 and *IPO90012*-MX12. The broad resistance to *M. graminicola* in this cultivar might be due to just one resistance factor for which virulence in the pathogen popu-

lation is rare. Studies on the genetics of resistance in wheat to *M. graminicola* indicate that monogenic, oligogenic and quantitative inheritance can be involved (21,57,88,132,133). In case the resistance in cv. Kavkaz/ K4500 1.6.a.4 would be largely quantitatively inherited, the detected virulence emphasises that pathogen populations may also adapt to that form of resistance. In that case quantitatively inherited resistance would not be necessarily durable. Obviously, abrupt circumvention of resistance by adaptation in the pathogen demonstrates the disadvantage of resistance conferred by one or a few genes with large effects, though quantitative resistance neither excludes specificity nor adaptation by pathogens (13,54,74,75,89,94). Even if wheat cultivars have not been reported to unequivocally succumb to new pathotypes of the fungus (56), the present and previous (61) studies demonstrated specificity, and thus the potential of the pathogen to circumvent resistance in the host. However, adaptation of *M. graminicola* to resistance in the host, by an increment of the frequency of individuals with appropriate virulence characteristics, might be relatively slow since it is strongly associated with the dissemination of such individuals. The rainsplash-dispersal of the asexual pycnidiospores during the growing season is relatively inefficient (119) as compared to the distribution of airborne propagules such as uredospores of rusts. Indeed, ascospores of *M. graminicola* are air-borne and efficiently disseminated (118). They are of prime importance for the establishment of the disease in the young crop, but most

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probably not for the rate of an epidemic in a developing crop. Sudden epidemics are often associated with mutational events from avirulence to virulence and extended distribution of such clones, and are particularly clearly demonstrated in pathogens with typically qualitative aspects of resistance and virulence or those that lack a generative stage (boom and bust) (13,54). In contrast, *M. graminicola* is a pathogen which is more characterized by quantitative aspects of resistance and virulence. In addition to that it has a functional sexual cycle and a relatively inefficient mechanism of spore dispersal during the growing season, hence a gradual deterioration of resistance rather than explosive epidemics should be expected. It is not surprising, therefore, that decline of resistance to *M. graminicola* remains often unobserved, and thus not reported, particularly when the commercial cultivation of cultivars is short. Nevertheless examples such as the Dutch cv.

Obelisk, which was considered as resistant when released in 1985, and is currently among the most susceptible cultivars, emphasize that adaptation in the pathogen population should not be under-estimated (7,66).

PATHOGEN ISOLATES with a high σ^2_P, as well as isolates with a low σ^2_P and a high overall P level, might carry relatively large proportions of specific virulence factors. Such isolates are considered to be of importance as tester isolates in breeding for durable resistance (94). Hence, breeding cultivars durably resistant to *M. graminicola* should be preceded by a thorough pathogen survey to enable the selection and employment of such isolates.

APART FROM the fact that similar conclusions applied to N, the difference between this parameter and P, as illustrated by dissimilarities of σ^2_P and σ^2_N, requires a comprehensive effort to elucidate its biological meaning.

CHAPTER THREE

COMPARATIVE SEEDLING AND ADULT PLANT EXPERIMENTS UNDER FIELD CONDITONS
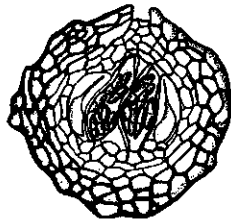
ABSTRACT

Fourteen Dutch *Mycosphaerella graminicola* isolates were studied for their virulence to 22 wheat cultivars in the seedling stage in an experiment which was performed according a completely randomized block design with three repetitions. Isolate x cultivar interactions were highly significant. Cluster analyses were applied to perform an unprejudiced selection of isolates with significantly different virulence characteristics for both disease parameters. These were retested in the seedling stage and used to inoculate two field experiments that were planted according to a split-plot design in 1992 and 1995. Overhead inoculations were conducted after flowering to avoid effects of tallness, hence these experiments were intended as monocyclic tests for virulence differences between the isolates. Significant isolate x cultivar interactions were determined for both disease parameters in each experiment, demon-strating specificity in the wheat-*M. graminicola* pathosystem in the adult plant stage under field conditions. The reproducibility of the adult plant data was high, although there was a significant year*cultivar*isolate interaction, which was primarily due to cv. Kavkaz and *M. - graminicola* isolate IPO001, as revealed from analyses of subsets excluding these entries. Genetic differences between the isolates were additionally evidenced by randomly amplified polymorphic DNA patterns (RAPDs), which also showed that no significant contamination of the inoculated plots with the natural *M. graminicola* population had occurred. Correlations between seedling and adult plant data were significant for *M. graminicola* isolate IPO323, but not for isolates IPO001 and IPO290. Hence, evaluation of resistance and virulence may require seedling as well as adult plant tests.

eptoria tritici leaf blotch is a disease of bread wheat and durum wheat, *Triticum aestivum* L. and *T. turgidum* ssp. *durum* L., respectively, caused by *Mycosphaerella graminicola* (Fuckel) J. Schröt. *in* Cohn (anamorph : *Septoria tritici* Rob. ex. Desm.), which was recently found to be a bipolar hetero-thallic fungus (65). The disease is of increasing importance in regions with a temperate climate, including Western Europe (100). Ascospores are consi-dered to be responsible for the esta-blishment of the disease in autumn and winter (104,118). However, their possible role in disease progress du-ring the growing season was only recently discussed by Kema et al. (65) who demonstrated the ability of *M. graminicola* to complete several gene-rative cycles per year. Hence, *M. gra-minicola* might be responsible for the establishment of different founder populations throughout the season, which is in accord with the vast genetic variation for molecular and virulence markers (10,61,63,81,82).

Specificity in the wheat-*M. gramini-cola* pathosystem was recently discus-sed by Kema et al. (61,63). They provi-ded circumstantial evidence for adap-tation to host species and cultivars, and suggested that the lack of con-sensus about this subject in literature might have been due to differences in the applied methodologies and en-

vironmental conditions. In addition, studies on genetic variation for virulence in *M. graminicola* were entirely based on seedling experiments under controlled conditions (32,33,61,63,134). Translation of such data to adult plants under field conditions, however, requires validation by comparative experiments. We report the occurrence of cultivar*isolate interactions in comparative seedling vs. adult plant experiments, which were conducted under controlled and field conditions, respectively.

MATERIALS AND METHODS

PLANT MATERIALS. Twenty-two bread wheat cultivars were used to study genetic variation for resistance in the seedling stage. The same cultivars were used in a field experiment in 1992, whereas a subset of five cultivars was selected for a field experiment in 1995 (Table 1). The majority of the cultivars has been of commercial importance in The Netherlands since the early thirties.

MYCOSPHAERELLA GRAMINICOLA ISOLATES. Fourteen Dutch *M. graminicola*

TABLE 1. Pedigree and cultivation period in The Netherlands of 22 bread wheat cultivars that were used to study genetic variation for virulence in 14 Dutch *Mycosphaerella graminicola* isolates in seedling and adult plant experiments.

Cultivar	Pedigree	Cultivation period
Carsten V	Dikkop/Criewener 104	1932-1957
Heines VII	Hybride à courte paille/Svälof's Kronen	1950-1967
Felix	Tassilo/Carsten//Carsten/Marquillo	1958-1973
Manella	Alba/Heine's VII	1964-1980
Joss Cambier	Heine's VII/Tadepi	1968-1978
Lely	Cebeco 30/Flevina	1969-1979
Clement[a]	Hope/Timstein//3xHeines VII/3/Riebesel 57-41/2xHeines VII/4/Cleo	1972-1980
Okapi[a]	Capelle Desprez/Carsten's Winterweizen III	1975-1990
Arminda	Carsten's 854/Ibis	1976-1990
Obelisk	Selection from a composite cross of 36 cultivars	1985-1993
Taurus	RPB 181-70D/Griffin	>1987
Ritmo	Hobbit/1320//Wizzard/3/Marksman/Virtue	>1992
Estica	Arminda/Virtue	>1993
Bussard[c]	Kranich/Maris Hunstman//Monopol	-
Vivant[a]	Boxer/Gawain	>1993
Rektor[c]	Kormoran/Monopol	-
Renan[c]	Mironovskaya 808/Maris Huntsman//VPM/Moisson//Courtot	-
Hereward[a]	Norman's sib/Disponent	>1993
Bon Fermier[c]	Gros bleu/Blé seigle	-
Veranopolis[b,c]	TCO/B2017-37	-
Kavkaz[a,c]	Lutescens 314H147/Bezostaja 1	-
SVP 72017[d]	Marzotto//Dippes Triumph/Mironovskaya 80	-

[a]Cultivars employed in field experiment 1995.
[b]Brazilian spring wheat.
[c]Cultivar which has not been cultivated in The Netherlands.
[d]Experimental wheat line.

TABLE 2. Dutch *Mycosphaerella graminicola* isolates that were employed to study genetic variation for virulence to seedlings and adult plants of 22 wheat cultivars

Isolate	Origin	Year
IPO235	Anjum, *cv*. Cebeco 148	1972
IPO290[a]	Flevoland, *cv*. Clement	1975
IPO281	Ebelsheerd, *cv*. Lely	1975
IPO295	Nagele, *id*.	1976
IPO304	Rusthoeve, *cv*. CB 314	1978
IPO323[a]	Brabant, *cv*. Arminda	1981
IPO333	Zuidzande, *cv*. Nautica	1981
IPO381	Heerle, *cv*. Marksman	1981
IPO394	's Heer Arendskerke, *id*.	1981
IPO439	Ebelsheerd, *id*.	1982
IPO89011	Barendrecht, ?	1989
IPO001[a]	Ulrum, F$_1$ population	1991
IPO003	Ulrum, *cv*. Obelisk	1991
IPO006	Wageningen, *cv*. Arminda	1991

[a] Isolates employed in field experiments.

isolates, selected over a period of approximately 20 years, were used to study genetic variation for virulence as expressed on seedlings (Table 2). Cluster analyses were applied to select a subset of three isolates (IPO001, IPO290, IPO323), which significantly differed from each other for virulence characteristics (Disease parameters: N; percentage leaf area with necrosis on primary leaves, P; percentage leaf area with pycnidia on primary leaves). These isolates were used to inoculate two field experiments in 1992 and 1995, respectively.

EXPERIMENTAL DESIGN AND DATA ANA-LYSES. Inoculum preparation, experimental conditions and procedures were similar to those described by Kema et al. (61,63). The seedling experiment was conducted according to a randomized block design with three repetitions. The selected subset of isolates was retested once to check the repeatability of the observed data.

The field experiments were conducted according to a split-plot design with two replications. In both experiments, *M. graminicola* isolates and a non-inoculated control were randomly allocated to main plots, and the cultivars were randomized within these main plots. The cultivars were planted to small fields (gross size 9 m^2, net size 2 m^2) which were separated by 3 m of winter barley within each treatment, and by 6 m of winter barley between the treatments. The field experiments were subjected to standard agronomical activities, except fungicide treatments. The experiments were set up as monocyclic tests for interactions in the adult plant stage and were therefore inoculated at growth stages ≥61 (141) to avoid effects of tallness (4,22). In order to provide favorable relative humidities in the crop an irrigation system was installed. The system was switched on prior to the first inoculation for 24 h at an interval of one minute irrigation every quarter of an hour, and was adjusted later to an interval of one minute irrigation every 30 minutes during day time.

The 1992 field experiment was inoculated on June 4 (2.3x10^6 spores. ml^{-1}), June 9 (2.0x10^6 spores.ml^{-1}), and June 22 (1.1x10^6 spores.ml^{-1}). On June 29, random samples from 10-15 plants of each plot in each treatment were taken for disease assessment on flag and second leaves of the main tillers. The 1995 field experiment was inoculated on May 24 (1.0x10^6 spores.ml^{-1}), May 29 (0.2x10^6 spores.ml^{-1}), and June 2 (0.12x10^6 spores.ml^{-1}). At June 22 ran-

dom samples, of 10-15 plants from each plot in each treatment, were taken for disease assessment on flag and second leaves of the main tillers. The first and second inoculations were conducted under favorable weather conditions, providing a natural humid environment for at least 48 h.

All statistical analyses were conducted by the Genstat 5 statistical package (41), using generalized linear models (GLM) with logit link and variance function proportional to M(100-M), where M is the mean disease parameter, non-parametric procedures and cluster analyses as described by Kema et al. (61,63). The cluster analyses were performed on complete seedling data sets, whereas the GLM on seedling data was restricted to the selected subset of isolates that was used to inoculate the field experiment. The adult plant data were corrected for natural disease levels in the control plots and statistical analyses were restricted to data collected from the flag leaves since inoculum deposition on the second leaf layer was partly intercepted by the flag leaves, which is a factor difficult to account for. Correlations between seedling and adult plant responses were calculated for each *M. graminicola* isolate to estimate the predictive value of observations at either growth stage.

DNA EXTRACTION AND RAPD ANALYSIS. To ensure that the observed disease severities were due to the inoculations with isolates *IPO001, IPO290, IPO323* a sample of diseased leaves was taken from each treatment in both field experiments in late august. Conidial cultures of these isolates and those

employed to inoculate the field experiment were stored at -80°C for comparison. DNA extraction and RAPD analysis, using primer OPA-20 (GTTGCGATCC, Operon Technologies Inc.), were conducted according to a protocol that was described previously (65).

RESULTS

SEEDLING EXPERIMENT. Analysis of the seedling experiment data revealed many significant differences between pathogen isolates and host genotypes for both disease parameters, which were summarized in dendrograms (Figs. 1 and 2). Significantly different groups for N and P did not contain the same cultivars or isolates (data not shown) as was observed previously (61). In contrast to the high N levels that were induced by the isolates, the P levels were low in the majority of the cultivars (Table 3). This was confirmed in an independent check experiment using the selected subset of three isolates (not shown).

FIELD EXPERIMENT 1992. The crop continued to be green until ca. 19 days after the first inoculation, which is considerably longer than in the seedling stage, followed by development of necrosis and pycnidia in a relatively short period of time. The responses were considered to be primarily due to the first inoculation, since the relative humidity (RH) in the crop upon that inoculation was >85% for 82 h, with a 4 hr reduction of RH at >70%, and a mean temperature of 18°C, with a minimum and a maxi-
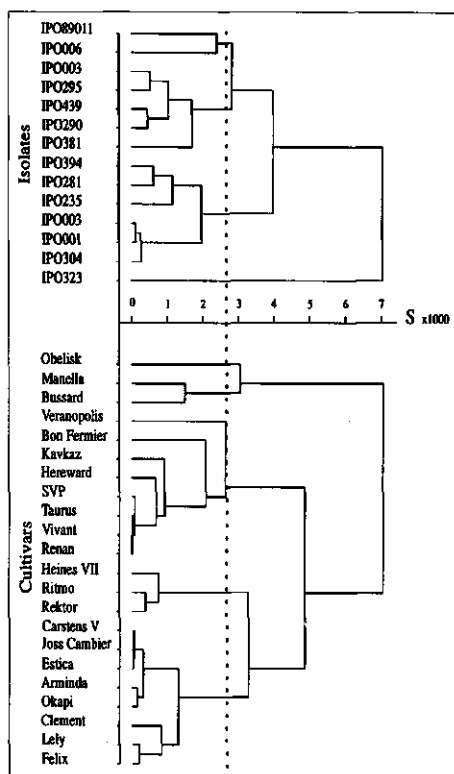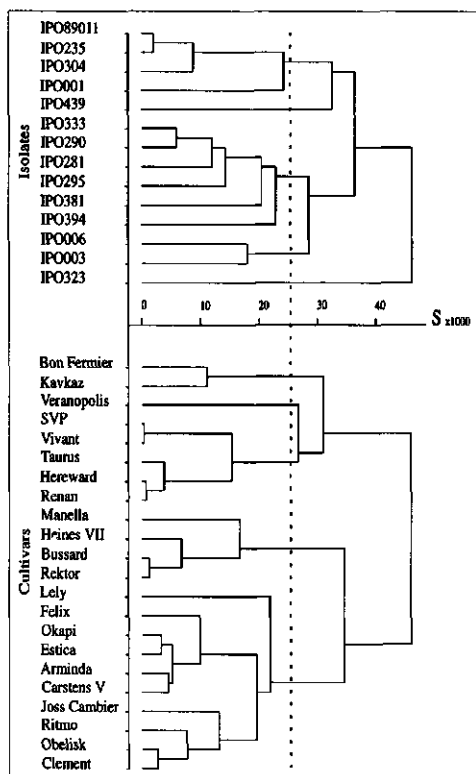
Fig. 1. Dendrograms of simultaneously clustered wheat genotypes (22) and *Mycosphaerella graminicola* isolates (14), based on N. The positions of the nodes correspond with the cumulative sum of squares for interaction between cultivars and isolates (S) on the horizontal axis. The area at the left of the dotted line represents non-significant differences at $P=0.05$, $S=26.5*10^3$ (for $P=0.01$, $S=28.4*10^3$).

Fig. 2. Dendrograms of simultaneously clustered wheat genotypes (22) and *Mycosphaerella graminicola* isolates (14), based on P. The positions of the nodes correspond with the cumulative sum of squares for interaction between cultivars and isolates (S) on the horizontal axis. The area at the left of the dotted line represents non-significant differences at $P=0.05$, $S=2.6*10^3$ (for $P=0.01$, $S=2,8*10^3$).

mum at 14°C and 25°C, respectively. Conditions after the second inoculation were sub-optimal, whereas the effect of the third inoculation can be ruled out since the plants developed their symptoms two days after it and were sampled only five days later. The N and P levels for the different isolates varied widely, and significant cultivar* isolate interactions for both disease parameters were observed (Table 3). Isolate *IPO323* appeared to be a differentiating isolate on the set of cultivars, whereas isolate *IPO290* showed a broad virulence. Both isolates were highly aggressive, particularly with respect to N, though cultivars Veranopolis, SVP 72017, Carsten V and Okapi were (relatively) resistant to isolate

TABLE 3. Comparison of responses, necrosis and pycnidia, in a seedling experiment and an adult plant field field experiment comprising 22 wheat cultivars and three *Mycosphaerella graminicola* isolates, which were conducted in 1992 in three and two replications, respectively[a].

Necrosis

	Seedling						Adult				
	IPO323		IPO290		IPO001		IPO323		IPO290		IPO001	
Veranopolis	12	ghij	27	opq	44	tuvw	0	a	3	abc	12	defg
Bon Fermier	9	cdefgh	41	tuvw	17	klmno	15	fghi	97	αβγ	20	ghij
SVP 72017	10	defghi	22	lmno	10	fghi	12	defg	58	qr	18	defgh
Taurus	4	bc	26	op	18	klmn	11	defg	77	rst	21	hij
Renan	8	cdefgh	37	rstu	24	mnop	3	ab	86	tuvwx	43	lmno
Lely	71	ζη	74	ζη	13	hijk	93	wxyzαβγ	93	xyzαβγδ	7	bcedf
Felix	88	θ	79	η	35	qrst	90	uvwxyzαβ	97	βγδ	52	op
Manella	69	βγδζ	46	wxy	10	ghi	88	uvwxyz	90	uvwxyzα	4	abcd
Obelisk	71	γδεζη	62	zαβ	31	pqr	98	γδ	99	δ	23	hij
Bussard	73	εζη	65	βγδε	25	nop	96	αβγδ	96	αβγδ	39	klmn
Joss Cambier	75	ζη	26	pqrs	6	bcdef	86	tuvwxy	90	uvwxyzα	17	fghi
Carsten V	62	zαβγδ	46	uvw	7	bcdefg	97	βγδ	58	pq	7	abcde
Estica	62	zαβ	40	stuvw	17	jklm	98	δ	89	uvwxyzα	16	fghi
Arminda	72	εζη	29	opq	10	fghi	91	uvwxyzαβ	92	uvwxyzαβ	12	efgh
Rektor	73	εζη	71	δεζη	31	pqrs	91	vwxyzαβ	89	uvwxyzα	27	ijk
Ritmo	56	xyz	28	op	24	op	83	stu	94	yzαβγδ	39	mno
Heines VII	65	αβγδε	45	vwx	15	hijk	85	tuvw	95	αβγδ	8	bcdef
Kavkaz	18	jklmn	63	zαβγ	25	nop	51	nop	96	αβγδ	23	jk
Hereward	1	a	15	ijkl	8	cdefg	8	bcdef	94	zαβγδ	34	jklm
Vivant	3	ab	10	efghi	5	bcdef	9	cdef	77	rst	84	stuv
Clement	56	yzα	41	stuvw	5	bcd	30	jkl	90	uvwxyza	9	cdef
Okapi	65	βγδε	38	rstuv	5	bcde	98	γδ	74	rs	11	defg

Pycnidia

	Seedling						Adult				
Veranopolis	0	abcdef	1	abcdef	11	lm	0	a	0	a	0	a
Bon Fermier	0	abcde	10	klm	0	abcdef	1	a	77	ijkl	3	a
SVP 72017	0	abcdef	1	abcdef	0	ab	0	a	31	e	3	a
Taurus	0	ab	3	efg	0	a	1	a	54	f	2	a
Renan	0	ab	2	bcdef	0	a	0	a	74	ghijk	21	c
Lely	4	fgh	4	ghij	2	bcdef	89	opq	88	opq	2	a
Felix	9	jkl	3	efg	0	abcdef	81	klmno	78	ijklmn	34	de
Manella	42	s	10	lm	0	a	81	jklmno	70	ghi	0	a
Obelisk	57	t	31	r	11	lm	93	pq	86	mno	10	ab
Bussard	40	s	15	no	0	a	94	q	81	jklmno	34	de
Joss Cambier	9	klm	1	abcdef	0	abcd	79	ijklmn	75	hijkl	5	a
Carsten V	12	lm	6	hijk	0	abc	78	ijklm	26	cd	0	a
Estica	11	lm	3	fg	0	a	85	mno	64	fgh	7	a
Arminda	18	op	2	cdefg	0	ab	89	opq	74	ghijkl	1	a
Rektor	26	q	12	mn	0	abcdef	82	lmno	70	ghi	12	ab
Ritmo	23	pq	5	ghi	6	hijk	79	ijklm	79	jklmn	8	a
Heines VII	22	op	6	ijk	0	a	79	jklmn	78	ijkl	1	a
Kavkaz	0	abcdef	3	hijk	1	abcde	49	de	87	nop	17	bc
Hereward	0	ab	3	efg	1	abcdef	0	a	81	jklmno	25	cd
Vivant	0	ab	1	abcdef	0	a	0	a	62	fg	3	a
Clement	12	lm	5	ghi	0	ab	22	c	77	ijkl	1	a
Okapi	17	no	2	defg	0	ab	71	ghij	27	cd	0	a

[a] Figures are backtransformed means of data, percentages necrosis and pycnidia, that were averaged over 10-15 primary leaves and flag leaves for the seedling and field experiments, respectively. Pairwise comparisons for seedling and adult plant data, respectively, were performed between all cells by approximate t-tests on the transformed scale. Significant differences (P<0.05) are indicated by different letters.

TABLE 4. Comparison of responses, necrosis and pycnidia, in two adult plant field experiments comprising five wheat cultivars and three *Mycosphaerella graminicola* isolates, which were conducted in two replications during each of the years 1992 and 1995[a].

	Necrosis					
	1992			1995		
	IPO323	IPO290	IPO001	IPO323	IPO290	IPO001
Kavkaz	51[a] f	96 ijk	23 de	18 bcd	93 ijk	13 bcd
Hereward	8 ab	94 ijk	34 ef	4 a	98 jk	17 cd
Vivant	9 abc	77 g	84 hi	8 abc	97 jk	8 abc
Clement	30 e	90 ijk	9 abc	24 de	98 k	7 ab
Okapi	98 jk	74 g	11 abc	91 ij	79 gh	11 abc
	Pycnidia					
	1992			1995		
	IPO323	IPO290	IPO001	IPO323	IPO290	IPO001
Kavkaz	49 cd	87 k	17 ab	3 a	69 ghi	1 a
Hereward	0 a	81 jk	25 bc	0 a	80 ijk	16 ab
Vivant	0 a	62 fgh	3 a	0 a	60 fg	2 a
Clement	22 ab	77 ijk	1 a	15 a	56 ef	0 a
Okapi	71 hij	27 bc	0 a	50 ef	44 de	0 a

[a] Figures are backtransformed means of data, percentages necrosis and pycnidia, that were averaged over 10 flag leaves and analyzed on logit scale. Pairwise comparisons for necrosis and pycnidia, respectively, were performed between all cells by approximate t-tests on the transformed scale. Significant differences (P<0.01) are indicated by different letters.

IPO290. Isolate IPO001 was rather avirulent compared to the aforementioned isolates. The cultivars Vivant, Hereward, Clement, Kavkaz and Okapi showed significant interactions with the three isolates for N and P and were therefore selected for the next field experiment, which was primarily conducted to verify these interactions.

FIELD EXPERIMENT 1995. The weather conditions following the first inoculation were particularly conducive to infection by *M. graminicola*. A high natural RH resulted in a leaf wetness period of 20 h. directly after inoculation, followed by a dry spell of 8 h and again leaf wetness during 17 h. The mean temperature during the first 48 h. after inoculation was 21.4 °C. The development of the disease was similar to the 1992 experiment, except that the final disease levels were generally lower, which might have been due to the relatively warm and dry summer (Table 4). The virulence and resistance characteristics were largely similar to the 1992 experiment, and once more highly significant cultivar*isolate interactions were observed for both disease parameters.

STATISTICAL ANALYSES. The cultivar* isolate interactions in the GLM analyses for both disease parameters in all experiments were highly significant, indicating specificity at both growth stages (Tables 3-5). Non-parametric

TABLE 5. Accumulated analyses of deviance of the disease parameters N and P in a seedling experiment and two field experiments. A selected subset of the seedling experiment was analyzed, which comprised all 22 wheat cultivars but only three of the 14 *Mycosphaerella graminicola* isolates. The field experiments in 1992 and 1995 comprised 22 and five bread wheat cultivars, respectively, inoculated with the three selected *Mycosphaerella graminicola* isolates.

Change		N		P
	df	MD[a]	df	MD[a]
Seedling experiment				
blocks	2	1693[b]	2	84[b]
cultivars	21	1355[b]	21	896[b]
isolates	2	10245[b]	2	5777[b]
cultivars*isolates	42	495[b]	42	143[b]
Error	2331	18	2312	8
Field experiment 1992				
blocks	1	67[b]	1	48[b]
cultivars	21	843[b]	21	983[b]
isolates	2	19965[b]	2	21771[b]
cultivars*isolates	42	693[b]	42	553[b]
Error	1272	17	1285	14
Field experiment 1995				
blocks	1	2[c]	1	36[c]
cultivars	4	226[b]	4	88[b]
isolates	2	8864[b]	2	5018[b]
cultivars*isolates	8	540[b]	8	478[b]
Error	281	10	283	8

[a]MD=Mean deviance.
[b]F-value highly significant (P<0.01).
[c]Not significant.

statistical procedures as described by Kema et al. (63) were applied on all data sets, and also revealed significant cultivar * isolate interactions (not shown). Correlations between N and P for the seedling and adult plant data (pooled for the 1992 and 1995 experiment) were not significant (not shown), which is in accordance with previous data (61,63).

A joint analysis of the 1995 data and the relevant 1992 data (subset of cultivars similar to the 1995 experiment) was performed to study the year effect (Tables 4 and 6). The year*cultivar*isolate interaction was significant for both disease parameters, though the size of this interaction for N was larger than for P, a phenomenon which was observed for almost all deviance components, and particularly for the year*isolate interaction (Table 6). Additional statistical analyses of subsets indicated that the significances of the year*isolate*cultivar and the year*isolate interactions were particularly due to the different response of cv. Kavkaz to isolate *IPO323* and the different performance of isolate *IPO001*, particularly on cv. Vivant.

Correlations between seedling and adult plant responses for each isolate were only significant for isolate *IPO323* (Tables 3 and 7). This was particularly true for N. For P, several cultivars such as Lely, Felix, Joss Cambier, Carsten V and Estica were, in spite of the significance, much more susceptible in the adult than in the seedling stage, a trend that was also observed for the other isolates.

RAPD ANALYSES. The non-inoculated controls in both field experiments were virtually free of disease on the flag leaves. Comparative RAPD analyses of isolates from the sporadic lesions on the flag leaves and from the inoculated plots suggested that the lesions on the

TABLE 6. Analyses of deviance of the disease parameters N and P to study the year effect in two adult plant field experiments conducted during 1992 and 1995. The selected subset of five wheat cultivars from the experiment in 1992 comprised the same cultivars as the experiment of 1995. Both experiments were inoculated with the same three *Mycosphaerella graminicola* isolates.

Change	df	N MD[a]	df	P MD[a]
yr	1	548[a]	1	442[a]
yr*block	2	3[b]	2	106[a]
isolate	2	11734[a]	2	8877[a]
cultivar	4	404[a]	4	286[a]
cultivar*isolate	8	1530[a]	8	1225[a]
yr*isolate	2	7052[a]	2	148[a]
yr*cultivar	4	126[a]	4	164[a]
yr*cultivar*isolate	8	222[a]	8	42[a]
Error	565	13	567	11

[a]F-value highly significant (P<0.01).
[b]Not significant.

TABLE 7. Correlations (R^2) between responses (N and P) of seedlings and adult plants[a] to three *Mycosphaerella graminicola* isolates.

Parameter	Isolates IPO323	IPO290	IPO001
N	0.83[**,b]	0.03[ns]	0.02[ns]
P	0.48[**]	0.08[ns]	0.0[c]

[a]Data of the two field experiments were pooled.
[b]ns=not significant, **=significant at P<0.01,
*=significant at P<0.05.
[c]Residual variance exceeds variance of the fitted variate (P).

flag leaves in the non-inoculated plots were due to the natural *M. graminicola* population (Fig. 3). RAPD patterns of isolations from the inoculated plots and the applied inoculum of isolates

IPO323, IPO290 and IPO001 were identical (Fig. 3).
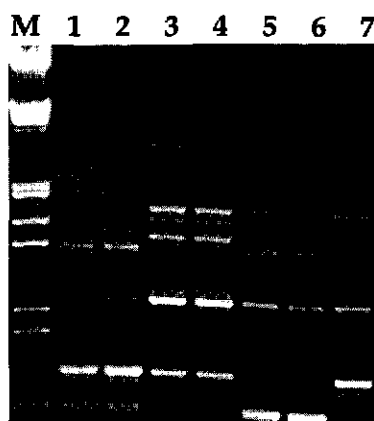
Fig. 3. PCR products of *M. graminicola* isolates. M: DNA marker, Lane 1: IPO001 inoculum, Lane 2: reisolation from field inoculated with IPO001, Lane 3: IPO290 inoculum, Lane 4: reisolation from field inoculated with IPO290, Lane 5: IPO323 inoculum, Lane 6: reisolation from field inoculated with IPO323, Lane 7: natural infection from the control plots.

DISCUSSION

THE RATIONALE for the current and previous studies (61,63) was to study whether specificity in the wheat-*M. graminicola* pathosystem occurs. From seedling data we recently concluded that specificity is an important characteristic of the pathosystem (61,63). The large cultivar*isolate interactions in the adult plant stage unequivocally confirmed that conclusion. Until now, virulence studies in *M. graminicola* did not consider adult plant experiments. Field evaluations of resistance to *M. graminicola* have been conducted and showed substantial differences between cultivars (12,17,103,115,127). However, these studies did not take into account possible genetic differences between

pathogen isolates, hence potential interactions between host cultivars and pathogen isolates, since they relied on the application of a single isolate, isolate mixtures or infections established by heterogeneous natural populations (12,17, 103,114,115,127).

THE CLUSTER analysis was mainly applied to perform an unprejudiced selection of isolates that showed significantly different virulence characteristics for N and P. The suggested genetic differences between the selected isolates IPO323, IPO290 and IPO001 were evidenced by RAPD data and confirmed by the adult plant experiments. Hence, cluster analysis appears to be an efficient tool to structure data large sets (18,61). In the seedling and adult plant stage N and P were not necessarily of similar magnitude, which was also demonstrated by these analyses that revealed dissimilarly assembled clusters of isolates or cultivars in dendrograms N and P.

COMPARISON OF seedling and adult plant data revealed some interesting differences. Isolate IPO323 was very different form the other isolates in that it differentiated the cultivars largely in a resistant group and a (moderately) susceptible group at both growth stages. Isolate IPO001, which was largely avirulent, particularly in the seedling stage. Isolate IPO290 was fairly avirulent when applied to seedlings, but highly virulent and aggressive toward adult plants. Therefore, the correlations between seedling and adult plant data for both N and P for the latter isolates were not significant. In general, adult plants appeared to be more susceptible than seedlings. Hence, our data provide evidence for the specific expression of resistance genes at the seedling stage but not at the adult plant stage. In wheat-rust pathosystems, some resistance genes are specifically expressed in the adult plant stage, and others are expressed in all growth stages (86,87, 102). Whether genes that control resistance to *M. graminicola* at the seedling stage are also responsible for adult plant resistance as observed in e.g. cvs. Bon Fermier, Taurus, Hereward and Vivant to isolate IPO323 is not known. If toxins are involved in the pathogenesis, as was recently suggested (66), the response of 'SVP 72017' is of interest. This experimental line had adequate resistance to all three *M. graminicola* isolates, and was included in the experiments since it had among the best levels of resistance to Fusarium head blight caused by *Fusarium culmorum* (122,123). Part of this resistance was due to a mechanism that either degraded or blocked the synthesis of the mycotoxin deoxynivalenol (122). Resistance to Fusarium head blight can be categorized in *i*) resistance to initial penetration, and *ii*) resistance to systemic fungal growth (112). Interestingly, resistance to *M. graminicola* appeared to be mainly due to a mechanism that restricted colonization (66).

THE CURRENT evidence for cultivar* isolate interactions in relatively small field experiments, accentuates the genetic variation for virulence in *M. graminicola*. Although the results of both experiments were largely similar, it is not surprising that the year* cultivar*isolate effect was significant

for a pathogen that is so sensitive to environmental conditions, of which particularly relative humidity is an important component (21,49,80,116, 117). The disease severities in 1995 were usually lower than in 1992, most clearly exemplified by cvs. Vivant and Kavkaz. 'Vivant' responded quite different with respect to N in the two field experiments. In 1992 it rapidly succumbed after inoculation, whereas in 1995 it remained largely healthy to isolate IPO001, whereas 'Kavkaz' responded less to all isolates. At present we cannot explain these observations, but it is likely that weather conditions have played a significant role. Both cultivars may be particularly sensitive to different temperatures, a phenomenon referred to as temperature-sensitive resistance in interactions with other pathogens (87). The inaccuracy of N emphasized that P is the more reliable parameter for disease assessment.

VIRULENCE FOR the majority of the cultivars was already present in the *M. graminicola* population before these were released. Isolate IPO290 was isolated in 1975 from cv. Clement but appeared to be highly virulent towards cvs. Hereward and Vivant. The latter cultivar covered the largest part of the total wheat acreage in The Netherlands from 1993 until 1995. The isolates IPO-323, isolated in 1981, and IPO290 were highly virulent towards cv. Arminda, which was the major Dutch wheat cultivar for almost a decade, with an acceptable level of resistance to *M. graminicola* when it was replaced by newer cultivars with adequate resistance, such as cv. Obelisk. In contrast, this cultivar was generally considered

as susceptible after its commercial life time. 'Obelisk' was bred using a composite cross strategy. Robinson (102) considered this procedure to come very close to the accumulation of 'horizontal resistance', which only failed if 'vertical resistance', or its effects, were not eliminated. Whether one agrees with this opinion or not, an obvious deduction would be that specificity in the wheat-*M. graminicola* pathosystem was apparently ignored. Selection pressure extended by a host genotype on a population of *M. graminicola* genotypes apparently results in increased levels of pathogenicity towards that genotype, but obviously takes longer on one cultivar than on the other. Recently, Ahmed et al. (1) provided evidence for the selective influence of host genotypes on *M. graminicola*. Consensus about specificity in the *M. graminicola*-wheat pathosystem, which was recently discussed by Kema et al. (61,63), was partly hindered by the contradiction between data obtained in experiments and the experience that wheat cultivars had not been reported to unequivocally succumb to new pathotypes of the fungus (56). However, such events are particularly clearly demonstrated in pathosystems with typically qualitative aspects of resistance and virulence. Kema et al. (63) argued that adaptation of *M. graminicola* to resistance in the host might have been overlooked since the process is probably rather slow, due to the characteristics of the pathosystem. The *M. graminicola* - wheat pathosystem appears to be characterized by a combination of quantitative and qualitative aspects of resistance and virulence. Recently, we showed

that *M. graminicola* populations are almost continuously capable to complete a generative cycle (65). The huge genetic diversity of *M. graminicola* populations (10,61,63,81,82,85) is a logical consequence of this observation. Hence, two important aspects of the pathosystem have to be considered to understand the aforementioned contradiction: *i*) host cultivars appear to be continuously exposed to extremely diverse populations, and *ii*) the frequency of pathogen genotyes with virulence characteristics matching resistance in the host will increase at a low rate. The first consideration differs widely from the regular experimental set-up where usually single isolates or simple mixtures are used (1,12,21,32,33,61,63, 103,134). Apart from the fact that wheat cultivars responded differently to isolate mixtures than to single isolates of *M. graminicola* (30,143), theoretical models have shown that specificity is difficult to prove if isolate mixtures are used (93,128). Hence, the fact that resistance in wheat cultivars does not seem to succumb rapidly to *M. graminicola* is not necessarily an appropriate argument against specificity, particularly since natural *M. graminicola* populations are by definition highly heterogeneous (10,65,81,82, 85). The question why interactions as observed in the present and other experiments (21,32,33,61,63) seem to be less evident from farmers fields is justified. We suppose that this is mainly due to: *i*) the general incompleteness of resistance in the host, which complicates evaluation of increased susceptibility, and *ii*) the diversity of natural *M. graminicola* populations, which masks the expression of specific factors.

GENERATION OF GENETIC VARIATION
IN
MYCOSPHAERELLA GRAMINICOLA

CHAPTER FOUR

DETERMINATION OF THE MATING SYSTEM
AND MOLECULAR ANALYSES
OF PROGENIES
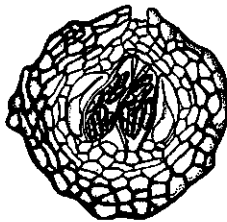
ABSTRACT

Monospore isolates of *Mycosphaerella graminicola*, which were considered to originate from one ascus were analysed by the polymerase chain reaction (PCR) with 32 RAPD primers. Eighteen primers revealed three classes of polymorphisms, which enabled a RAPD-based tetrad analysis. Four pairs of isolates resulting from a single diploid nucleus were determined. A procedure to cross these isolates was developed to investigate the mating system. Three of six crosses were successful, and segregation of mating types in accordance with the tetrad analysis, strongly points to a bipolar heterothallic mating system in *M. graminicola*. Random ascospore progenies from the successful crosses, each comprising 54 isolates, were studied with three primers to determine the mode of inheritance of the RAPD markers. Mendelian segregation and recombination of RAPD markers were observed in all progenies.

Septoria tritici leaf blotch is an important fungal disease of bread wheat and durum wheat, *Triticum aestivum* L. and *T. turgidum* (L.) Thell. ssp. *durum* L., respectively, in regions with a temperate climate, and becomes increasingly important in North-Western Europe (100,131). The disease is caused by the ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt. *in* Cohn (anamorph: *Septoria tritici* Rob. ex Desm.), which was described for the first time in New-Zealand by Sanderson (108). Since then, *M. graminicola* was also reported in several other wheat producing areas of the world (40,79,113). The air-borne ascospores are considered to be the most important source of primary inoculum that establishes the disease, whereas splash-dispersed pycnidio spores are believed to be the major propagules for progression of disease during the growing season (104,109,113,118,119).

The genetic variation of several markers in *M. graminicola* is vast. McDonald and Martinez (81) identified 22 haplotypes among 93 isolates from a single wheat field, and Boeger et al. (10) identified different genotypes even within one lesion, whereas different conidia from the same pycnidium had identical genotypes. Kema et al. (61,63) recently described genetic variation for virulence among 80 isolates and provided evidence of specificity for host species and host cultivars.

The large degree of genetic variation among *M. graminicola* isolates points to a more important role of ascospores in the epidemiology of septoria tritici leaf blotch as hitherto expected (85,119). Nevertheless, the mating system and the role of the sexual cycle for generating genetic variation in the pathogen remained to be resolved. We report a procedure to cross selected *M. graminicola* isolates, characterized the mating system and analysed segregation of molecular and morphological markers in the progenies.

MATERIALS AND METHODS

PATHOGEN ISOLATION PROCEDURE. Dutch farmers fields in Wageningen and Kraggenburg, which were planted to the major wheat cv. Vivant, were sampled between July 1994 and August 1995 for diseased leaves, wheat debris (stubble and leaves) and infected volunteer plants to investigate the occurrence of *M. graminicola*.

Collected samples were soaked in tap water for one hour. Leaf sheaths and leaves with distinct black fructifications were selected and placed on wet quadrants of filter paper ($\varnothing$ 9 cm) in the lids of Petri-dishes. Excess of water from the plant parts and filter paper quadrants was removed with dry filter paper just prior to closing the Petri-dishes, which were filled with water agar. The Petri-dishes were placed up-side down, and the lids of the were turned 90° every 15-20 min. Thus, pseudothecia were allowed to discharge ascospores for *ca.* 60 min. in each Petri-dish. The lids with filter paper and plant parts were subsequently replaced by regular lids and ascospores were allowed to germinate overnight at 22°C in an incubator. Groups of spores were considered to originate from the same ascus when located in an isolated position. Germinating ascospores were isolated under a dissecting microscope, and individually transferred to Campbell's ® V8 vegetable juice agar plates. After colony formation spore masses of each isolate were collected and preserved for further analysis at -80°C. Suspensions of spores from the same isolates were stored over silicagel at -20°C.

IN PLANTA CROSSING PROCEDURE. Attempts to cross *M. graminicola* isolates *in vitro* were unsuccessful (G.H.J. Kema, *unpublished*). Therefore, we decided to achieve crosses between *M. graminicola* isolates by developing an *in planta* procedure, using isolates from ascospores that were considered to originate from the same ascus. Conidial cultures of the isolates were individually incubated for five days in Erlenmeyer flasks with liquid yeast-glucose medium in a reciprocal shaker at 17°C. Conidia were allowed to sediment for several hours, the liquid medium was removed by aspiration, and the resulting pellets were resuspended in 100 cc volumes of tap water. Combinations of two isolates were prepared to inoculate 10 day old seedlings of the susceptible cv. Obelisk with a cotton swap. Environmental conditions during incubation were as described previously (61). Fourteen days after inoculation necrosis levels of up to 50% of the primary leaves and traces of pycnidia had developed. Henceforward, plants from each isolate mixture were transplanted to separate buckets, which were covered with fitting polyvinyl chloride (PVC) rings with coarsely darned PVC nets to allow exposure to external environmental conditions, but preventing the loss of leaf material. The buckets were placed outside on March 8th 1995. On March 21st many pycnidia had developed on the primary leaves. Samples from each bucket were subjected to the aforementioned ascospore isolation procedure.

MOLECULAR ANALYSES. Conidial cultures of single *M. graminicola* ascospores

were individually incubated as described before. The spores were pelleted by centrifugation and lyophilized. Total genomic DNA was extracted using a protocol adapted from Vilgalys and Gonzalez (135). The DNA concentration was estimated visually by comparing intensities of the samples with a standard gel electrophoresis marker (phage λ digested with *Hind*III and *Eco*RI), and eventually diluted to 1 ng.μl^{-1}. DNA aliquots of 100 μl were incubated according to the protocol of White et al. (137) in a Perkin Elmer DNA Model 9600 thermal cycler for the polymerase chain reaction (PCR). Each aliquot contained 60.7 μl MilliQ water, 10 μl (10x) Taq buffer (200mM Tris-HCl, pH 8.4, and 500mM KCl), 3 μl 50mM MgCl$_2$, 10 μl 2mM dNTPs, 0.24 μl Taq polymerase (5 units.μl^{-1}), 10 μl primer (10 ng.μl^{-1}) and 6 μl DNA (1ng.μl^{-1}), which was replaced by 6 μl MilliQ in the controls. PCR started with one incubation of 4' at 94°C, followed by 45 cycles of 1' at 94°C, 2' at 38°C and 3' at 72°C. Electrophoresis of each PCR sample was performed on horizontal 1% agarose gels containing 0.1 μg.μl^{-1} ethidium bromide for 4 h at 4V.cm^{-1}. Thirty-two decamer primers (Operon Technologies Inc.) were evaluated for detection of appropriate polymorphisms, and eventually primers OPA-8 (GTGACGTAGG), OPA-9 (GGGTAACGCC) and OPB-6 (TGCTCTGCCC) were selected for the analysis of random ascospore progenies.

PCR products from the parental isolates *IPO94265* and *IPO94266* were ligated with vector pGEM-T (Promega) and electroporated into *Escherichia coli* DH5α. Two clones of each transformation were labelled with α-^{32}P-dATP by random prime labelling, and used as probes in dot-blot and Southern hybridizations (107).

RESULTS

FIELD SAMPLES. Ascospores (Fig. 1A) were discharged from stubble and volunteer plants collected in the field in October 1994. The majority of ascospores were discharged within 30 min. after closing the Petri-dishes. Overnight incubation allowed all ascospores to germinate. Initial colony formation of *M. graminicola* continued by the development of additional extensions from the ascospores (Fig. 1B), and eventually resulted in typical colonies of the anamorph *S. tritici*. Sixty-one isolates were obtained, including sets of ascospores that might have originated from the same ascus since they were close to each other without other ascospores in the proximity.

DNA POLYMORPHISMS. RAPD analysis of sets of ascospores with 32 primers revealed polymorphisms within sets. One such set contained eight isolates, *IPO94265-IPO94272*, which had been discharged in complete isolation from other spores. Eighteen primers revealed three classes of polymorphic patterns in this set (Table 1), whereas 14 other primers did not. This enabled us to group the isolates in four pairs. Isolate *IPO94272* showed deviating RAPD bands for all 32 primers, suggesting that it originated from another ascus (Fig. 2). Hence, isolates *IPO94265* to *IPO94271* were used to develop an *in planta* crossing procedure.
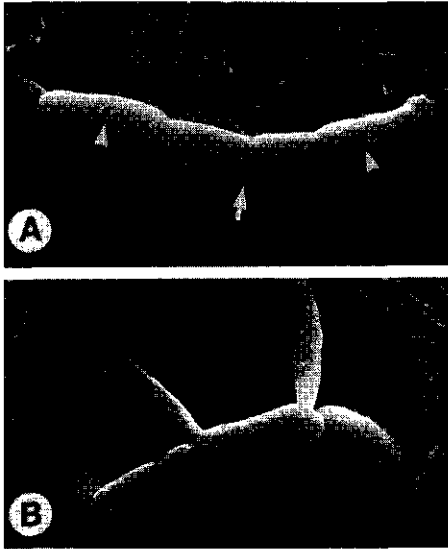
Fig. 1. A,B. Scanning electron micrographs of *Mycosphaerella graminicola* ascospores. **A.** A germinating two-celled ascospore on water agar, note the ascospore septum (arrow) and the thick germtubes extending from both terminal ends (arrowheads) (5900 x). **B.** Early colony formation by a budding ascospore on water agar (5900 x).

IN PLANTA CROSSES. Mixtures of isolate IPO94265 and each of the isolates IPO94266-71 were used in the *in planta* crossing procedure. Ascospores were discharged from leaves that were inoculated with isolate mixtures of IPO-94265 and either isolate IPO94266, IPO94268 or IPO94269. No ascospores were discharged from the other combinations (Table 2). In total, 351 progeny isolates were recovered from the three successful crosses. This observation is in accordance with the aforementioned pairs of ascospores (Table 1), and demonstrates, therefore, the bipolarity of the mating system in *M. graminicola*. We propose the assignment of alleles MAT1-1 and MAT1-2 (139) to the mating type locus in *M. graminicola*. Hence, allele MAT1-1 is assigned to isolate pairs IPO94265-IPO4267 and IPO94270-IPO94271, and allele MAT1-2 to isolate pair IPO94266-IPO94269 and isolate IPO94268.

GROWTH TYPES. The growth types, either yeast-like or mycelial (60), of the

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TABLE 1. Tetrad-analysis of *Mycosphaerella graminicola* isolates IPO94265-IPO94272, which were assumed to originate from the same ascus, based on 19 RAPD polymorphisms revealed with 18 primers.

	M. graminicola isolates							
Class	*IPO94265*	*IPO94266*	*IPO94267*	*IPO94268*	*IPO94269*	*IPO94270*	*IPO94271*	*IPO94272*
I (N=6)[a]	A	A	A	B	A	B	B	C
II (N=6)[b]	A	B	A	B	B	A	A	C
III (N=7)[c]	A	B	A	A	B	B	B	C

[a]Primers OPA-4, OPA-9, OPA-12, OPA-13, OPA-16 and OPG-14.
[b]Primers OPA-5, OPA-12, OPA-14, OPB-6, OPH-5, and OPH-14. Note that primer OPA-12 produced polymorphisms in class I as well as class II.
[c]Primers OPA-7, OPA-8, OPA-11, OPA-17, OPB-8, OPG-6 and OPG-7.
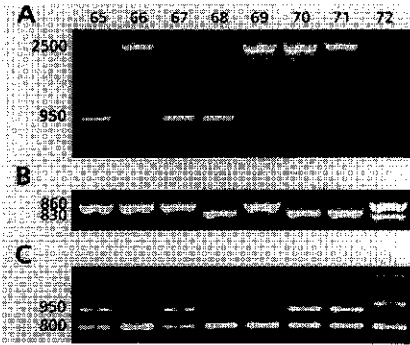
Fig. 2. A-C. RAPD patterns of eight *Mycosphaerella graminicola* isolates *IPO94265-IPO94272* that were supposed to originate from the same ascus. RAPD products obtained with A. primer OPA-8, B. primer OPA-9, and C. primer OPB-6.

individual isolates of two progenies, *IPO94265* x *IPO94268* and *IPO94265* x *IPO94269*, were determined on V8 agar, and were found to segregate in a 1:1 fashion (Table 2). Isolates that had initially a yeast-like growth type easily shifted to mycelial growth in later asexual generations, indicating that the

control of this marker is not stable.

INHERITANCE OF RAPD MARKERS. Random ascospore progenies, comprising 54 isolates from each cross, were each analysed with two of the primers OPA-8, OPA-9 and OPB-6, which revealed clear polymorphisms among the parental isolates (Fig. 2).

CROSS 1 : *IPO94265xIPO94266*. Amplification of parent isolate *IPO94265* with primer OPA-8 resulted in a 950 bp product, whereas isolate *IPO94266* had a 2500 bp product (Fig. 3A). The progeny of the cross between these isolates revealed a 1:1 segregation for the parental 950 bp and 2500 bp products (Fig. 3A, Table 3). One isolate from this cross contained both bands and was therefore considered to be a recombinant.

Analysis of the parents with primer OPB-6 resulted in a 950 bp and a 800 bp product in isolate *IPO94265*, and a 800 bp product in isolate *IPO94266*

TABLE 2. The number of progeny isolates that were recovered from crosses among seven *Mycosphaerella graminicola* ascospore isolates (*IPO94265-IPO94271*), and the segregation ratios for their growth types on V8 agar.

Cross	Result	Progeny isolates			χ^2	P
		Σ^a	YL[b]	M[c]		
IPO94265 x *IPO94266*	+[d]	113	nd[e]	nd		
IPO94265 x *IPO94267*	-	0				
IPO94265 x *IPO94268*	+	119	60	59	0.01	0.9-0.95
IPO94265 x *IPO94269*	+	119	53	66	1.42	0.2-0.3
IPO94265 x *IPO94270*	-	0				
IPO94265 x *IPO94271*	-	0				

[a]Σ=Total number of recovered isolates.
[b]YL=Yeast-like growth type.
[c]M=Mycelial growth type.
[d]+=successful cross, -=unsuccessful cross.
[e]nd=not determined.
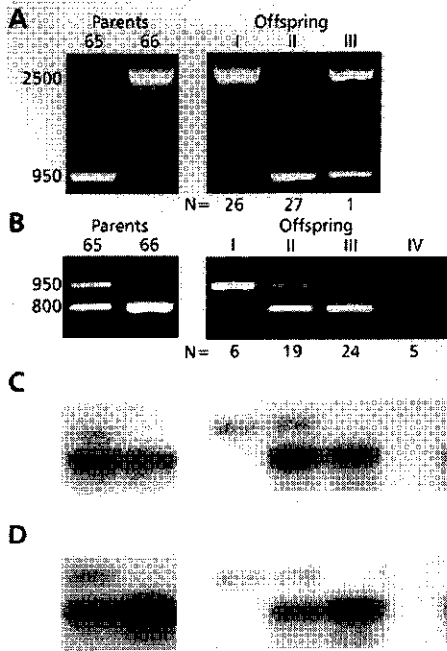
Fig. 3. A-D. Ascospore progeny analysis of 54 random isolates obtained from the cross between *Mycosphaerella graminicola* isolates *IPO94265* x *IPO94266*. **A**. RAPD patterns of parental and offspring isolates with primer OPA-8. **B**. RAPD patterns of parental and offspring isolates with primer OPB-6. The number of each offspring genotype, categorized in parental patterns (II, III) or recombinant patterns (I, IV), is shown underneath. **C**. Southern blot of the gel in **B** with a 800 bp probe derived from isolate *IPO94265*, and **D**. with a 800 bp probe derived from isolate *IPO94266*.

(Fig. 3B). The segregation of these products in the progeny was according to independent segregation of three characters; a 950 bp and two 800 bp products (Fig. 3B, Table 3), indicating that the 800 bp OPB-6 RAPD marker in *IPO94265* and *IPO94266* was on different loci. Dot-blot analyses of the clo-

ned 800 bp products from both isolates showed that they were homogeneous as well as homologous (not shown). Southern analysis on the offspring isolates of this cross confirmed these data. Probes derived from either parent hybridized to all offspring isolates that contained a 800 bp band, which indicated that the parental 800 bp products had a high degree of similarity (Figs. 3C-D). This was substantiated by digestion of the amplification products with eight restriction enzymes that revealed identical patterns for both parental 800 bp products (not shown). Apparently, there was also some homology between the 950 bp product and the 800 bp products, as demonstrated by weak cross hybridization with both probes (Figs. 3C-D).

CROSS 2 : *IPO94265xIPO94268*. Amplification of parent isolate *IPO94268* with primer OPB-6 resulted also in a 800 bp product. Amplification of the progeny with primer OPB-6 revealed a pattern different from cross 1, since the 800 bp product was present in all isolates, which suggests that isolates *IPO94265* and *IPO94268* carry the same allele for the 800 bp OPB-6 marker. The 950 bp product that originated from *IPO94265* segregated in a 1:1 ratio (Fig. 4A, Table 3).

Amplification of parental DNA with primer OPA-9 resulted in a 860 bp product in isolate *IPO94265* and a 830 bp product in isolate *IPO94268*. Amplification of progeny DNA showed three groups, whereas four were expected if both markers were inherited independently. A group of 12 isolates had neither product and a second group of 14
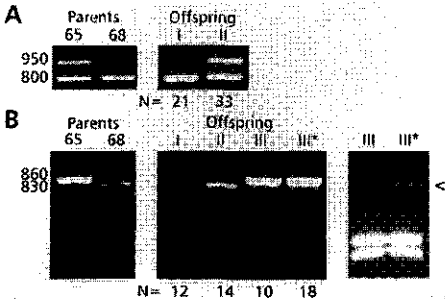
Fig. 4. A,B. Ascospore progeny analysis of 54 random isolates obtained from the cross between *Mycosphaerella graminicola* isolates IPO94265 x IPO94268. **A.** RAPD patterns of parental and offspring isolates with primer OPB-6. **B.** RAPD patterns of parental and offspring isolates with primer OPA-9. The number of each offspring genotype, categorized in parental patterns (II, III) or recombinant patterns (I, III*), is shown underneath. The discrimination between recombinant patterns III and III* by digestion with restriction enzyme *Hae*III is depicted in the right panel.

isolates had only the 830 bp product (Fig. 4B). Hence, the third group (Fig. 4B, offspring type III), which comprised 28 isolates, probably contained isolates with either only the 860 bp product or two indistinguisable 830 bp and 860 bp products. Thus, genotypes that only contained the 860 bp product or those that contained both the 830 bp and the 860 bp products could not be separated (Fig. 4B). The 860 bp product of isolate IPO94265 could be digested with restriction enzyme *Hae*III, resulting into two fragments of 480 bp and 380 bp, whereas the 830 bp product from IPO94268 was not digested. Therefore, the PCR product of all type III progeny isolates was digested with this restriction enzyme, resulting in discrimination between either genotype (Fig. 4B, digests III and III*), and

thus confirmed independent segregation of the OPA-9 RAPD markers in the progeny isolates (Table 3).

CROSS 3 : IPO94265xIPO94269. Amplification of progeny DNA from this cross with primers OPA-8 and OPB-6 revealed a similar segregation of the PCR products as observed in the progeny of cross IPO94265xIPO94266 (Table 3).

LINKAGE GROUPS. Possible linkage between the RAPD markers was determined by comparing the frequencies of the possible combinations of these RAPD markers. The frequencies of the RAPD marker combinations in the three crosses were not significantly different. Thus the 1:1 segregation for the two OPA-8 and OPA-9 markers in the entire progenies was also observed in each subset of the progeny as defined by the OPB-6 genotypes. Hence, there appeared to be no linkage between RAPD markers produced by different primers.

EPIDEMIOLOGY. We used ascospore discharges from field samples as an indication for favourable weather conditions for ascosporogenesis. The first natural *M. graminicola* ascospore discharge from field samples was observed on April 6th (Table 4). Ascospores from the successful crosses were discharged from samples that were collected on April 12th. Hence, a five weeks exposure (from March 8th-April 12th) to the outdoor environment was appropriate to complete ascocarp formation. Continued analysis of lower leaf samples, which were collected during winter, spring and

TABLE 3. Analysis of observed and expected genetic segregation ratios for target sites of three RAPD primers in progenies (N=54) of three crosses between *Mycosphaerella graminicola* isolates.

Cross	Primer	Parental products	Progeny isolates				χ^{2e}	df[f]	P
			Σ^a	$G_E{}^b$	$N_O{}^c$	$N_E{}^d$			
1 *IPO94265xIPO94266*	OPA-8	950 bp	54	950	27	27	0.04	1	0.8-0.9
		2500 bp		2500	26	27			
				950+2500	1	0			
				-	0	0			
	OPB-6[g]	800 bp	54	800	24	19.9	1.33	3	0.7-0.8
		950 bp		950	6	6.6			
				800 + 950	19	19.9			
				-	5	6.6			
2 *IPO94265xIPO94268*	OPA-9	830 bp	54	830	14	13.5	2.59	3	0.3-0.5
		860 bp		860	10	13.5			
				830 + 860	18	13.5			
				-	12	13.5			
	OPB-6	800 bp	54	800	21	27	2.67	1	0.1-0.2
		950 bp		950	0	0			
				800 + 950	33	27			
				-	0	0			
3 *IPO94265xIPO94269*	OPA-8	950 bp	54	950	27	27	0.0	1	1.0
		2500 bp		2500	27	27			
				950 + 2500	0	0			
				-	0	0			
	OPB-6[f]	800 bp	54	800	21	21.3	0.56	3	0.9-0.95
		950 bp		950	6	6.8			
				800 + 950	19	21.3			
				-	8	6.8			

[a]Σ=Total number of isolates tested.
[b]G_E=Expected genotypes in the progenies.
[c]N_O=Number of observed genotypes in the progenies.
[d]N_E=Number of expected genotypes in the progenies.
[e]χ^2 calculation excluded all genotypes with N_E=0.
[f]df=degrees of freedom.
[g]Since both parental isolates produced a 800 bp fragment, expected and observed frequencies are of phenotypes.

summer of 1995 from a farmer's field in Wageningen, demonstrated asco-spore discharges of *M. graminicola* from April 6th until May 4th, and from June 9th until July 28th (Table 4). In contrast, ascospores were not dischar-ged from flag leaves that were collec-ted in July 1994 in Kraggenburg

TABLE 4. Discharge of ascospores of *Mycosphaerella graminicola* from samples collected during winter, spring and summer of 1995, in a naturally infected farmers field planted to cv. Vivant.

Month	02	03	..	..	..	..	04	..	..	..	05	..	..	..	06	..	..	..	..	07	..	..	..	08
Date	25	01	09	16	23	30	06	13	20	27	04	12	19	26	02	09	16	23	30	06	14	21	28	04
	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	-

(North-East polder), whereas numerous ascospores were discharged from both wheat debris and volunteer plants that were collected from the same field in October 1994.

DISCUSSION

WE REPORT the first crosses among selected *M. graminicola* isolates. Sexual reproduction in *M. graminicola* appeared to be controlled by a bipolar heterothallic mating system, as demonstrated by three successful crosses among six pairings of isolates that were considered to originate from the same ascus. This assumption, based on the isolated position of eight ascospores in the applied ascospore discharge system, was supported by 19 RAPD polymorphisms revealed with 18 primers. Although the noncylindrical ascus, and hence the random arrangement of ascospores in the ascus, hampers or even prevents regular tetrad-analysis in *M. graminicola*, RAPD analyses enabled us to arrange isolates *IPO94265-IPO94271* in the four pairs resulting from a single diploid nucleus.

ANALYSIS OF the progenies revealed that morphological and RAPD markers were inherited according to Mendelian genetics. Yeast- and mycelial growth types segregated in a 1:1 fashion.

Analysis of segregation in the progenies of crosses *IPO94265* x *IPO94266* and *IPO94265* x *IPO94269* using primer OPA-8 revealed only one recombinant offspring isolate, whereas the remaining isolates showed the parental RAPD patterns. Hence, the 950 bp and 2500 bp markers generated with OPA-8 appear to be linked in repulsion, with a recombination percentage less than 1% (1 out of 108). Linkage between DNA markers in *M. graminicola* was also observed by McDonald and Martinez (83,84). They postulated 17-18 chromosomes in seven *M. graminicola* isolates, and observed that three RFLP probes hybridized to the largest chromosome (83). Since we analysed each progeny with two RAPD primers, the frequency distributions of RAPD marker combinations in the three progenies were compared to test for linkage between RAPD markers produced by different primers. The frequency distributions were found to be similar, hence suggesting independent inheritance of the markers produced by the OPA-8, OPA-9 and OPB-6 primers. Application of only three RAPD primers in our study revealed, therefore, five linkage groups, which may represent five chromosomes of the *M. graminicola* genome.

THE INDEPENDENT assortment of the

-91-

OPB-6 RAPD markers was particularly of interest with respect to the 800 bp product that was present in all parental isolates. Analysis of the progenies of crosses IPO94265 x IPO94266 and IPO94265 x IPO94269, indicated that the 800 bp products from both parents segregated independently, which was not observed in the progeny of IPO94265 x IPO94268. Southern and restriction analyses indicated that the 800 bp products in isolates IPO94265 and IPO94266 are identical. Nevertheless, both markers segregated independently, which is an indication that they are on different loci in the parental isolates. This is most probably due to translocation, possibly to another chromosome. On the basis of our current data we cannot exclude rearrangement within the same chromosome, such as an inversion. However, this is not likely since it would probably have disturbed the independent segregation of both loci, which matched exactly the expected frequency of 25% of the offspring isolates lacking the 800 bp product. We postulate that the same translocation is present in isolate IPO94269, but absent in isolate IPO94268. This interpretation would be in accordance with the RAPD-based tetrad-analysis.

MCDONALD AND Martinez also found M. graminicola isolates that were partially diploid at an RFLP locus defined by probe pSTL53. They suggested this to be due to either a translocation resulting from a parasexual recombination event, or to a transposition mediated by a LINE-like element (84). In our study, RAPD markers were inherited according to Mendelian genetics.

Therefore, the translocated OPB-6 800 bp markers in isolates IPO94266 and IPO94269, which were inherited independently in progenies of crosses between IPO94265 and these isolates, will be duplicated in 25% of these progenies. Hence, there is evidence that duplication in M. graminicola can result from genetical recombination of translocated sequences, in addition to the aforementioned possibilities suggested by McDonald and Martinez (84). We are currently testing this hypothesis.

AN IN vitro procedure to cross Phaeosphaeria nodorum isolates (45,46), was not successful for M. graminicola (G.H.J. Kema, unpublished). Therefore, we exposed inoculated plants to the natural environment to achieve crosses between M. graminicola isolates. Halama and Lacoste (45) analysed all possible combinations of two isolates (28) originating from the same ascus, whereas we combined one isolate with the remaining isolates from the same ascus. Both procedures provided evidence for the heterothallic nature of the mating systems in these two fungi. We successfully repeated some of the aforementioned crosses, and additional crosses between less related isolates were also successful. The crossing procedure, therefore, proved to be reliable, hence M. graminicola is now amenable to regular genetic analysis, which will enhance the perception of genetic variation (61,63,81,82,85) in this pathogen. The progenies derived from crosses between less related isolates will be useful in the construction of a genetic map of M. graminicola, which will support molecular genetic analysis

of virulence. Similar developments in research on *Magnaporthe grisea* (76,120, 121), *Tapesia yallundae* (20,26) and *Leptosphaeria maculans* (38,99) has contributed significantly to the elucidation of the genetic control of virulence and resistance in the relationships of those pathogens with their respective hosts.

OUR PROCEDURE for crossing *M. graminicola* isolates showed that the pathogen is able to complete a sexual cycle within five weeks under conducive conditions. It appears that such conditions occur throughout the season, as demonstrated by analysis of field samples in the laboratory, which showed that *M. graminicola* ascospores were ejected from wheat debris and volunteer plants in autumn, from young wheat plants in spring and from the lower leaf layers of adult plants in summer. Moreover, our crosses were conducted using seedlings under highly varying weather conditions in March/April, October/December and November/January.

THE IMPORTANCE of these epidemiological observations is substantial, since it demonstrates that *M. graminicola* is able to complete several sexual cycles per season. Thus, progress of septoria tritici blotch after establishment of the disease does not merely depend on splash dispersal of asexual conidia (104,119), but it also includes a polycyclic process of airborne propagules, with consequently a different genetic constitution of each generation. The relative importance of the polycyclic sexual process compared to the dissemination of asexual spores is unknown. However, ascospores are air-borne, and their discharge is initiated by changes in relative humidity, which are not necessarily due to rain but also to dew and fog (109). Hence, contrary to the splash-dispersal of the asexual propagules the frequency of opportunities for ascospore release, and as such their contribution to the genetic structure of the population, might be much higher than previously thought. An obvious suggestion would be that ascospores are largely responsible for primary infections on different leaf layers, thus establishing different founder populations, whereas pycnidiospores for secondary infections. Population genetical studies of McDonald et al. (85) support this idea. Although there is consensus on the involvement of ascospores in establishing the disease in young wheat crops (118), their contribution to disease progress was ignored in recent epidemiological studies (104,119).

ANOTHER IMPORTANT inference from our study is that a wheat crop seems to be exposed to several genetically different and highly diverse ascospore generations throughout the growing season. Hence, breeders that expose their material to natural infections should be aware that the pathogen population is not only highly diverse, but also discontinuous within one season and over seasons, probably also with respect to virulence. The vast genotypic diversity in *M. graminicola*, even on a single leaf (10,61,63,81,82), and the occurrence of large isolate* cultivar interactions in seedling and adult plant experiments (64) suggest that the quantitative appearance of disease severity in the *M. graminicola-*

wheat relationship is perhaps due to the presence of a mixed population of genotypes with qualitative differences. Thus, quantitativeness in the wheat-*M. graminicola* relationship might be an apparent quantitativeness. Such a situation could lead to erroneous interpretations of resistance levels, *i.e.* confusing partial resistance with incomplete resistance provided by several major genes, and hence to a low selection efficiency in breeding programmes, as was observed in other crops and screening studies with race mixtures (93,94). Therefore, an improved understanding of the genetic control of virulence in *M. graminicola*, is an indispensable prerequisite for the development of effective breeding strategies.

HISTOPATHOLOGY

CHAPTER FIVE

ANALYSIS OF THE PATHOGENESIS OF
MYCOSPHAERELLA GRAMINICOLA
IN A RESISTANT AND
SUSCEPTIBLE HOST
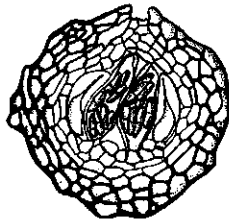
ABSTRACT

Cellular aspects of the pathogenesis of *Mycosphaerella graminicola* in a susceptible and resistant wheat cultivar were studied by light microscopy (LM), and scanning (SEM) and transmission electron microscopy (TEM). Experiments were designed as time-sequence studies in two replications with sampling dates at 12, 24 and 48 hours post inoculation (hpi), and 4, 8, 10, 12, 14 and 16 days post inoculation (dpi). A separate experiment was performed to quantify the mycelial biomass in cvs. Shafir and Kavkaz/K4500 l.6.a.4 at the afore-mentioned intervals using a double antibody sandwich ELISA assay for *M. graminicola*.

The germination frequency of *M. graminicola* conidia was high in both compatible and incompatible interactions, but the infection frequency was low. Infection was strictly stomatal but appeared to be a random process, since many germ tubes crossed stomata without penetrating them. Some germ tubes formed branched structures close to or on top of stomata. These structures were small compared to the size of stomata, were formed irregularly and were not significantly correlated with successful penetrations of the host. Multiple penetrations of stomata occurred regularly. Hyphae of *M. graminicola* were already observed in the substomatal cavities at 12 hpi, and at 48 hpi, hyphae had reached the nearest mesophyll cells.

In the compatible response, colonization was fairly limited until 8 dpi. Hyphae grew intercellularly and in close contact with the mesophyll cells. During the 10-12 dpi interval, extensive host cell death occurred, which induced further colonization and, eventually pycnidium formation in substomatal cavities. Initial and further colonization had marked effects on the number and size of the chloroplasts in the compatible interaction. Nevertheless, leaves remained green until approximately 10 dpi.

The resistance response was primarily characterized by very limited colonization, mostly in the vicinity of the substomatal cavity.

Quantification of the mycelial mass with ELISA revealed similar mycelial quantities in cvs. Shafir and Kavkaz/K4500 l.6.a.4 until 8 dpi. After 8 days, the mycelial quantity developed exponentially in cv. Shafir, but did not significantly increase in cv. Kavkaz/K4500 l.6.a.4.

M ycosphaerella graminicola (Fuc-kel) J. Schröt. *in* Cohn (ana-morph: *Septoria tritici* Rob. ex Desm.) is a fungal pathogen, which causes septoria tritici leaf blotch on bread wheat (*Triticum aestivum* L., AABBDD, 2n=42) and durum wheat (*T. turgidum* ssp. *du-rum*, AABB, 2n=28). The disease occurs particularly in temperate high-rainfall environments (131), and the symptoms are characterized by necrotic blotches, which contain varying densities of

pycnidia, the asexual fructifications containing the conidia.

The teleomorph is considered to be largely responsible for the over sum-mering of the disease in regions where crop rotation is a common agricultural practice, whereas the anamorph would mainly contribute to disease develop-ment during the growing season (118, 119). However, it was recently shown that *M. graminicola* is able to complete several generations of ascospores after the establishment of the disease in

autumn, a finding of considerable epidemiological importance (65). The recently determined heterothallic mating system (65) provides an explanation for the wide genetic variation in *M. graminicola*, and particularly for its potential to adapt to host species and cultivars (31,33,61,63, 81,82).

Nevertheless, resistance breeding is considered to be an effective strategy to control the disease (131). However, virtually nothing is known about the mechanism of resistance to *M. graminicola* in wheat cultivars (16,50,136). Resistance breeding might, therefore, benefit from a comprehensive study of the pathogenesis in compatible and incompatible interactions of wheat and *M. graminicola*, which was the subject of the present study.

MATERIALS AND METHODS

PLANT MATERIALS AND *M. GRAMINICOLA* ISOLATES. Based on previous studies (61) the bread wheat cvs. Shafir and Kavkaz/K4500 l.6.a.4 were selected for the study of compatible and incompatible responses to *M. graminicola* bread wheat isolate *IPO87016*, respectively. In addition, bread wheat isolate *IPO-235* and durum wheat isolate *IPO-86022*, and the durum wheat cv. Volcani 447 were occasionally used for comparison.

EXPERIMENTAL PROCEDURES AND CONDITIONS FOR MICROSCOPY. For LM, leaf materials were fixed under low vacuum for 24 h in 5% glutaraldehyde in a 0.02 M phosphate buffer. The samples were dehydrated in a graded ethanol series and embedded in Technovit 7100® resin and sectioned (2 μm)

with a Jung 2040 microtome equipped with Ralph glass knives. The sections were collected on glass slides, stained with toluidine blue for 10 min and photographed using a Zeiss Axioplan microscope equipped with an MC100 camera using bright field and differential interference contrast optics. Nuclei in conidia were stained with 4,6-diamidino-2-phenyl indole (DAPI, 2.5 μg.ml^{-1} in McIlvain's buffer, pH 4.4). Leaf material for SEM was fixed, critical-point dried with CO_2 and observed using a Jeol 35C scanning electron microscope. For TEM, leaves were fixed in 5% glutaraldehyde in a 0.02 M phosphate buffer, post-fixed and stained in 2% osmium-tetroxyde in 0.05 M sodium-cacodylate, dehydrated in an alcohol series, embedded in Spurr's standard resin and viewed using a Jeol 100CX transmission electron microscope.

Ten to 15 plants were grown in a line in square 7x7 cm pots, which were placed in controlled walk-in climate chambers with pre- and post-inoculation conditions that were similar to those described earlier (61). Quantitative inoculations were conducted by spraying suspensions of conidia (1×10^7 conidia.ml^{-1}, 30 ml per isolate, supplemented with two drops of Tween 20® surfactant) on the test cultivars (10 days old), which were placed on a turn table, adjusted to 15 rpm, in an isolation hood equipped with a pressure driven cleaning device and interchangeable atomizers in order to avoid contaminations. Plants were left for 1 h at a laboratory bench to dry. Primary leaves that were to be sampled for microscopical observations were then positioned in small transparent plastic

boxes (126), and re-inoculated along 1 cm of the leaf with 10-12 1-2 μl drops of inoculum, and were incubated for 12-48 h at a light intensity of approximately 3 $\mu E.s^{-1}.m^{-2}$. Control plants were treated with water.

From each isolate-cultivar combination the twice-inoculated sections of the primary leaves were sampled at different intervals: 12, 24 and 48 hours post inoculation (hpi), and 4, 8, 10, 12, 14 and 16 days post inoculation (dpi). The sampled plants and additional checks were left in the climate chamber for normal disease assessment (necrosis and pycnidium formation scored as percentages on the primary leaves, N and P, respectively) at 21 dpi. The experiments for light microscopy (LM, including differential interference contrast; DIC), and scanning (SEM) and transmission electron microscopy (TEM) were performed in two replications for each sampling, with five leaves per replication.

An additional SEM experiment was performed using cv. Shafir and isolate IPO87016 in two replications to quantify the penetration frequency and to investigate the association of penetration with the formation of appressorium-like structures. Inoculated leaves were harvested and fixed at 48 hpi and stomata were scanned and classified in four categories: i) without hyphal clusters, ii) with hyphal clusters, without penetration, iii) with hyphal clusters, penetration and appressorium-like structures, and iv) with hyphal clusters, penetration but no appressorium-like structures.

EXPERIMENTAL PROCEDURES AND CONDITIONS FOR MYCELIAL QUANTIFICATION.

A proprietary double antibody sandwich enzyme-linked immuno-sorbent assay (ELISA) kit based on a polyclonal antibody to a soluble metabolite of *M. graminicola* (DuPont, Stevenage, UK) was used to quantify the mycelial biomass of *M. graminicola* isolate IPO-87016.

Eight pre-germinated seeds of cvs. Shafir and Kavkaz/K4500 1.6.a.4 were sown in a diagonal line across 5x5 cm pots containing 125 cm^3 of John Innes 2 compost and grown at 21 °C to growth stage 11 (125) with a 16 h light period each day. The pots were arranged in six blocks of 14 pots within a controlled growth cabinet, each block consisting of two rows of seven pots. Cultivars and positions of pots to be harvested at different times were randomized within rows. Harvests of the first leaves of all plants in one pot of each cultivar from each block were made immediately before inoculation (controls). The remaining six pots of each block were simultaneously inoculated with *M. graminicola* isolate IPO-87016 on a rotary turntable at 16 rpm (15 ml inoculum of 10^7 conidia.ml^{-1}). Pots were enclosed in polythene bags for 48 h after inoculation to allow infection to take place. Primary leaves were harvested at 3, 6, 8, 10, 12 and 14 dpi for quantification of mycelium.

The leaf samples were kept at -20 °C until assayed. Leaves taken from two adjacent blocks were thawed, cut into 2-3 mm pieces and homogenized in 2.5 ml proprietary extraction buffer. Samples were allowed to stand for 1 h and were subsequently centrifuged at 9000 g for 5 min to sediment plant debris. The supernatant was used according to the protocol of the manufacturer of the

ELISA kit. Duplicate assays were made of each sample except at 3 dpi. The samples of cv. Shafir from 12 dpi and 14 dpi contained more antigen than the most concentrated standard. Hence, these levels were extrapolated from the 10x diluted samples. Positions of samples and standards on the micro-titre plate in which the assay was run were fully randomized. The experiment was analysed using the generalized linear model facilities in Genstat (41). Variances between replicates were not significantly larger than between duplicate wells containing the same extract. The mean square values between replicates were used to estimate the standard errors.

RESULTS

MACROSCOPICAL OBSERVATIONS. Controls for disease assessment remained apparently healthy until 8 dpi, after which necrosis developed and pycnidia were formed. Necrosis usually started from the leaf tips. Final disease levels after inoculation with *M. graminicola* isolate *IPO*87016 were 89% necrosis and 86% pycnidia on cv. Shafir and 5% necrosis and 1% pycnidia on cv. Kavkaz/K4500 1.6.a.4 at 21 dpi. The response of cv. Shafir to isolate *IPO*235 was characterized by relatively high necrosis (40%), but sparse pycnidia formation (5%). These observations were comparable to previous data (61).

Parts of primary leaves left on plants after sampling at 12 hpi and 24 hpi, thus with sub-optimal incubation periods, usually did not develop symptoms. All leaves of cv. Shafir in the other treatments, which were

appropriately incubated for 48 h, developed necrosis and pycnidia whereas those of cv. Kavkaz/K4500 1.6.a.4 did not. The controls of both cultivars remained free of disease throughout the experiment.

GERMINATION AND PENETRATION (Fig. 1). The frequency of non-germinated conidia was usually very low and often all observed conidia on both cultivars had germinated, usually from both ends. Chemo- or thigmotropism with respect to stomatal structures in the leaves was probably not involved, since germ tubes regularly crossed stomata or grew along the guard cells without entering the stomatal aperture (Fig. 1A). Germ tubes were often branched and commonly developed into clusters of hyphae aggregated in stomatal depressions of the leaves. Penetrations were strictly stomatal, direct penetration of the epidermis were not observed. They were achieved either by single germ tubes (Figs. 1B, E) or by the clusters of germ tubes or young mycelium, which resulted in multiple penetrations (Fig. 1F). Penetration was sometimes linked with the formation of appressorium-like structures on the stomatal aperture (Figs. 1C-D). However, in an additional experiment the frequencies of penetrated stomata with and without the presence of appressorium-like structures were not found to be significantly different (Table 1). The penetration frequency in this experiment was 25%, but it appeared to be much lower in the other experiments.

COLONIZATION (Figs. 2-4). Colonization during the compatible response of cv.
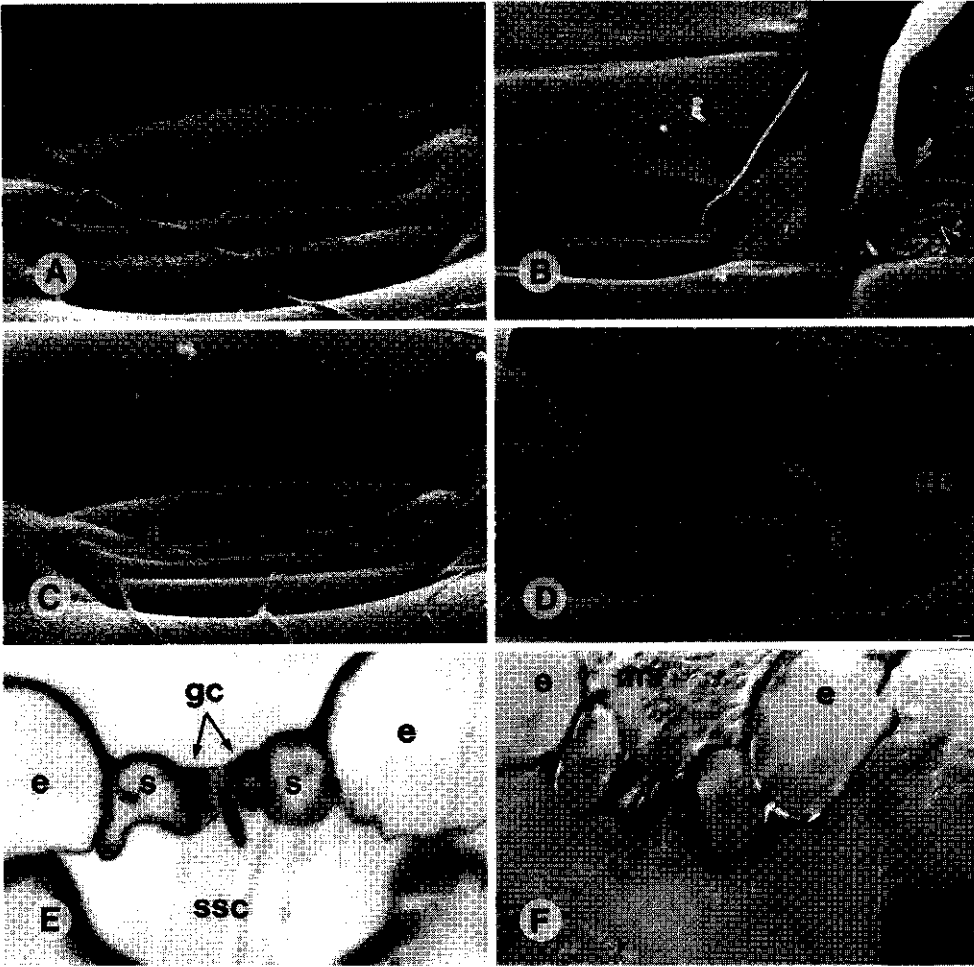
Fig. 1. Scanning electron and light micrographs of *Mycosphaerella graminicola* germ tube growth and penetration in cv. Shafir. A, Germ tubes of isolate *IPO87016* crossing the stomatal aperture without penetration at 48 hpi, x1550. B, Penetration of isolate *IPO235* of a stoma observed at 12 dpi. Note the flattened appearance of the germ tube, which is caused by closing of the guard cells (arrows), x2030 (inset x35000). C and D, Appressorium-like structures of *IPO87016* on guard cells at 48 hpi, x3125 and x3515, respectively. E, Penetration of a stoma by *IPO87016* at 48 hpi. Note the septum in the germ tube (arrow), x1050. F, Mycelial stroma or hyphal cluster on outside of stoma giving rise to multiple penetrations of *IPO86022* observed at 4 dpi. Note the hyphae (arrows) in the substomatal cavity and the starch granules in the chloroplasts (arrowheads), x1050.

Abbreviations for all figures: cl=chloroplast, cw=cell wall, e=epidermall cell, gc=guard cell, is=intercellular space, m=mesophyll cell, ms=mycelial stroma, nu=nucleus, s=subsidiary cell, ssc=substomatal cavity, wb=woronin body.

TABLE 1. Penetration frequency of germ tubes of *M. graminicola* isolate *IPO87016* on cv. Shafir and its association with appressorium-like structures (ALS) on stomata as observed by SEM at 48 hpi.. Values are totals of two replications.

	Σ^a
Without hyphal clusters	20
With hyphal clusters	
without penetration	88
with penetration + ALS	14[b]
with penetration - ALS	22[b]

[a]Σ=number of scanned stomata.
[b]Values for +ALS and -ALS are not significantly different, $\chi^2 = 1.87$.

Shafir infected with isolate *IPO87016*, and the effects on the number and shape of cellular organelles of the host were compared with the controls (cv. Shafir inoculated with water) at each sampling date. Since the controls did not deviate from each other at these time intervals, the control of 16 dpi is shown in Fig. 2A. In cv. Shafir, mycelium was observed in substomatal cavities from 12 hpi. At 48 hpi, hyphae with clearly visible vacuoles and nuclei reached and continued to colonize the mesophyll intercellularly, usually in close contact with the cell walls (Fig. 2B). During the 48 hpi - 8 dpi interval, mycelial growth remained strictly intercellular but gradually increased in quantity. At 8 dpi, mesophyll cell walls had an irregular wrinkled appearance and sometimes collapsed (Fig. 2C), a phenomenon that had considerably increased at 10 dpi (Fig. 2D). Hyphal growth in the mesophyll remained intercellular until cell death (Figs. 2C-H). Walls of cells in the colonized area were no longer stained by toluidine blue, particularly at 8-12 dpi (Figs. 2E, F). Rapid, massive host cell death, with the mycelium largely attached to the

Fig. 2. Light, scanning, and transmission electron micrographs of colonization during the compatible interaction between cv. Shafir and *Mycosphaerella graminicola* isolate *IPO87016*. A, Water-inoculated control at 16 dpi. Note the regular shape of the mesophyll cells and their organelles, x313. B, Intercellular growth between mesophyll cells just beyond the substomatal cavity at 48 hpi. Note the changed shape of the chloroplasts (arrows) and the vacuoles in the hypha (arrowheads), x625. C, Cross section through a primary leaf at 8 dpi. Note the intercellular growth of the hyphae (arrows), the wrinkled shape of the mesophyll cell walls, and the strong condensation of chloroplasts (intensely stained). For comparison of chloroplast stainability see Figs. 2A and 2D-F. Some mesophyll cells are on the verge of collapse (asterisks), x250. D, Intercellular growth (arrows) at the point of cell collapse at 10 dpi. The stainability and shape of the chloroplasts (arrowheads), which fill nearly the whole cell lumina, has changed compared to 8 dpi (Fig. 2C) and the control (Fig. 2A). The vascular bundle and bundle sheath cells appear to be affected, x625 (see also Fig. 2H). E and F, Overview (x250) and detail (x625), respectively, of a cross section through a primary leaf at 12 dpi. Note that hyphae (arrows) grow in close contact with the mesophyll cell walls, which are destained when being in contact with mycelium (see also Fig. 3A), and only clearly visible when they are adjacent. In F, hyphae (arrows) are in close contact with mesophyll cells, and chloroplasts (arrowheads) are swollen, hardly stained and comparable to those shown in Fig. 2D. G, Scanning electron micrograph of intercellular growth of hyphae at 12 dpi. The hyphae grow in close contact with the cell walls, attached to the cell wall by an extracellular matrix (arrows). Chloroplasts are vaguely visible through the cell wall (arrowheads), x6250. H, Transmission electron micrograph of intercellular growth of hyphae at 10 dpi. Note the degenerate chloroplasts and the intact cell wall, x10,000.

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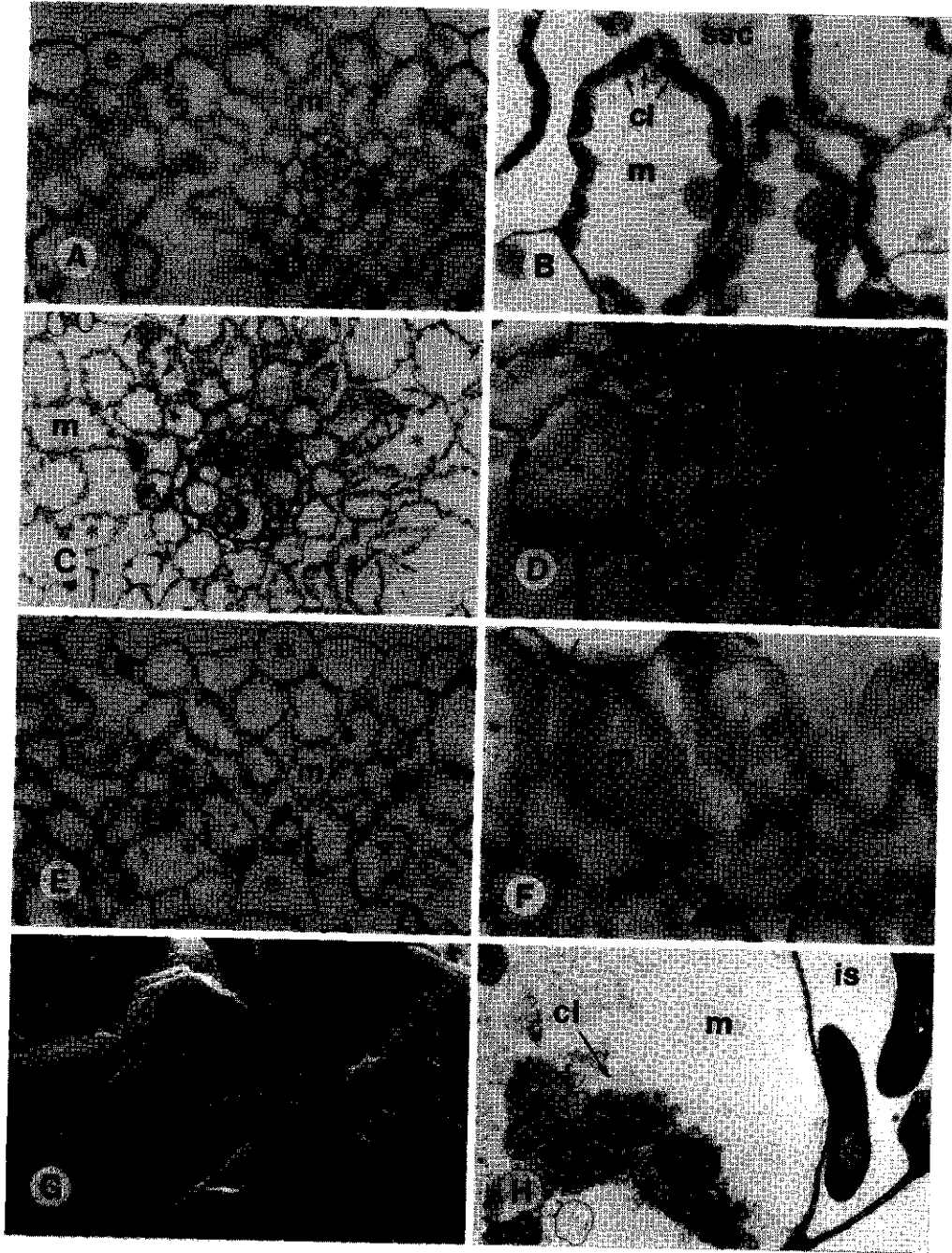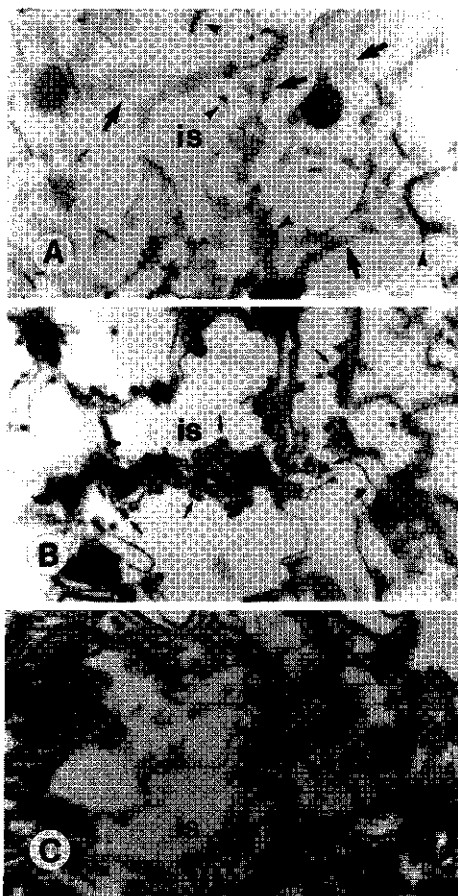
Fig. 3. Mesophyll cell collapse and its effect on mycelial proliferation of *Mycosphaerella graminicola* isolate *IPO87016* in cv. Shafir. **A,** Beginning of mesophyll cell collapse at 12 dpi. Note the irregular shape of the cells (arrows), the destained cell walls, which are in close contact with hyphae (arrowheads) and the hardly stained chloroplasts, x625. **B,** Massive mesophyll cell collapse at 14 dpi. Many hyphae (arrows) are found in the vicinity of the degenerated cells (see also Fig. 5C), x625. **C,** Colonization at 16 dpi. Advanced stage of mesophyll cell collapse, x625.

degenerated cell walls, was observed during the 10 dpi - 12 dpi interval (Fig. 3A, B see also Fig. 5C). This was

Fig. 4. Light micrographs of the incompatible and intermediate responses of cvs. Kavkaz/K4500 l.6.a.4 and Shafir toward *Mycosphaerella graminicola* isolates *IPO87016* and *IPO235*, respectively. **A,** Intercellular hypha (arrow) observed in cv. Kavkaz/K4500 l.6.a.4 at 10 dpi. The shape of the mesophyll cells and the number and size of the chloroplasts are similar to those observed in the controls and significantly dissimilar from those observed in cv. Shafir at the 8-10 dpi interval (see Figs. 2C-D), x313. **B,** Overview of a cross section of a primary leaf of cv. Shafir inoculated with isolate *IPO235* at 12 dpi. Colonization (arrows) is still fairly limited, but several cells are at the verge of collapse (asterisks). Note the irregular shape of the mesophyll cells and the large starch granules (arrowheads) in the chloroplasts, x313. **C,** Details of mesophyll cells of cv. Shafir at 14 dpi with isolate *IPO235*. Note the intercellular mycelium (arrows), the intensely stained chloroplasts (arrows), and the starch granules that appear to be released from the chloroplasts (arrowheads), x940. **D-F,** Details of chloroplast alteration in cv. Shafir at 10 dpi with isolate *IPO235*. Note that none of the mesophyll cells is in contact with or close to hyphae of the fungus. **D,** Small intensely stained and condensed chloroplasts (arrowheads), x400. **E,** Transitional zone showing mesophyll cells with intensely stained chloroplasts (arrowheads) and cells with swollen hardly stained chloroplasts (arrows), x313. **F,** Swollen hardly stained chloroplasts (arrowheads) of cells at the verge of collapse, x625.

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followed by mycelial proliferation, apparently resulting from the release of nutrients from the mesophyll cells after their collapse (Figs. 3B, C).

The shape of chloroplasts changed significantly during the 48 hpi - 12 dpi interval. Compared to the controls (Fig. 2A) they condensed and were,
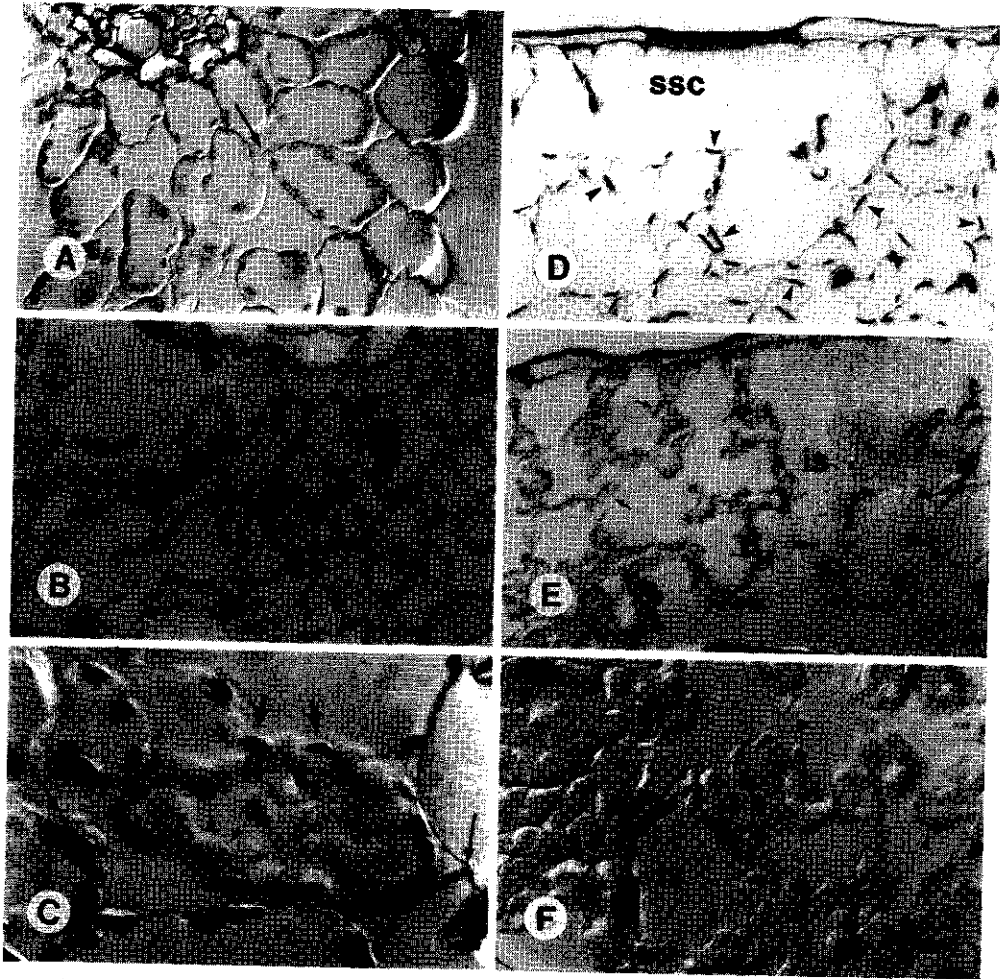
together with the nucleus, re-located toward the cell wall, probably as a result of the enlargement of the central vacuole (Figs. 2B-C). However, just prior to cell collapse chloroplasts seemed to expand again (Figs. 2D-F).

Compatible responses of the durum wheat cv. Volcani 447 to isolate *IPO-86022* (not shown) were analogous to the pathogenesis of isolate *IPO87016* in cv. Shafir.

The incompatible response of cv. Kavkaz/K4500 1.6.a.4 to isolate *IPO-*

87016 was primarily characterized by virtual lack of colonization. Hyphae were only occasionally observed between mesophyll cells, and only in the vicinity of substomatal cavities (LM observations; 3.5% and 22% of fungus-containing sections at 8 and 10 dpi, respectively). In contrast to observations at these stages in cv. Shafir after inoculations with isolates *IPO87016* and *IPO235*, the appearance of chloroplasts in mesophyll cells of cv. Kavkaz/K4500 1.6.a.4 was similar to

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Fig. 5. Light and transmission electron micrographs of asexual fructification of *Mycosphaerella graminicola* isolate *IPO87016* in cv. Shafir. **A**, Initiation of a pycnidial development under the stomatal aperture at 12 dpi. Note the intercellular hyphae (arrows), x625. **B and C**, Formation of a pre-pycnidium under the stomatal aperture (arrows) after massive mesophyll cell collapse at 10 and 14 dpi, respectively. Note the plasmolysis in the guard cells, subsidiary cells and epidermal cells in **B**, and the restricted distribution of mycelium in **C**, which is still largely confined to the cell walls of collapsed cells (arrowheads), x6000 and x625, respectively. **D**, Mature pycnidium in the substomatal cavity at 14 dpi. The ostiole of the pycnidium is located under the stomal slit. The pycnidial wall consists of a layer of conidiophores (arrowheads), each of which can sequentially produce conidia (see also Fig. 5A). Note that both epidermal cells remained uncolonized, x625.

the controls (Fig. 4A). There was no evidence for compartmentalization, hypersensitive responses or for processes related to cell wall strengthening by polyphenolic compounds or lignin (stained by Toluidine bleu).

Resistant responses of cv. Shafir to isolate *IPO86022* (not shown) appeared to be different from those of cv. Kavkaz/K4500 1.6.a.4 to isolate *IPO-87016*, as it involved slightly more colonization and incidental alterations of mesophyll cell walls.

Inoculation of cv. Shafir with isolate *IPO235* revealed a similar cytological picture with respect to isolate *IPO-87016*, though cv. Shafir is more resistant to isolate *IPO235*, and was less colonized (Fig. 4B). The chloroplasts contained clearly identifiable starch granules (Fig. 4B-C), which seemed to be released from the chloroplast just prior to cell collapse (Fig. 4C). At 10 dpi, the appearance of chloroplasts was notably different from the controls. They were either condensed and intensely stained or swollen and destained. However, these responses seemed to be provoked by soluble compounds produced by *M. graminicola*, since there was usually no mycelium in the vicinity of the cells with these defects (Figs. 4D-F).

PYCNIDIUM FORMATION (Fig. 5). Young pycnidia initials of isolate *IPO87016* were already observed from 8 dpi onward in the substomatal cavities of cv. Shafir (Fig. 5A). Thereafter, diverse stages of pycnidium formation were observed. Young initials evolved to mycelial stromata, typically located underneath the stoma (Fig. 5A, see also 2E), and developed into pre-pycnidia
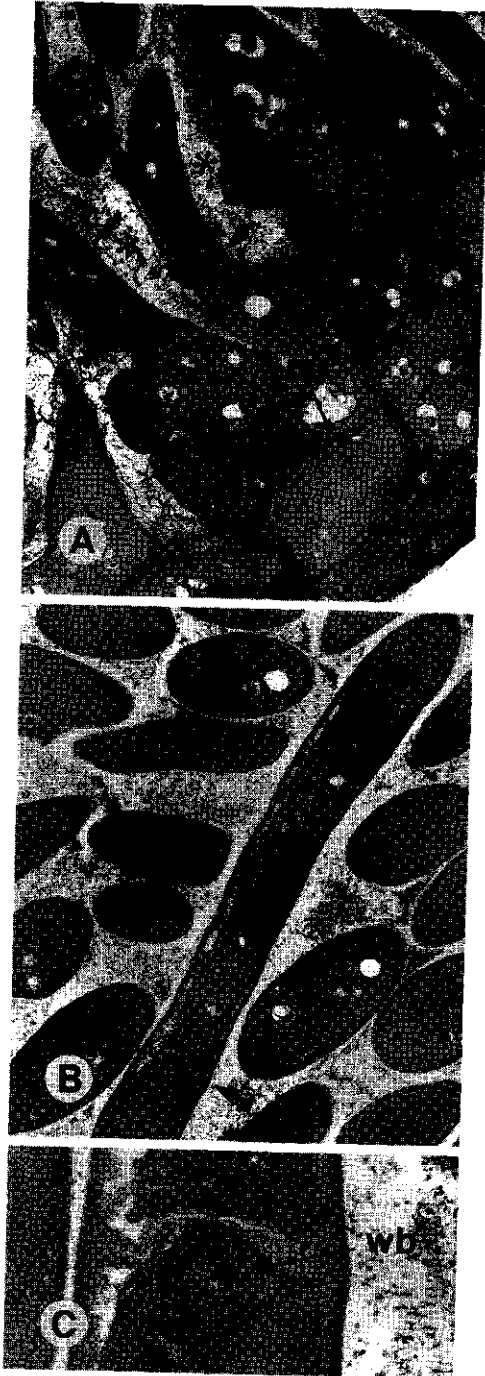
Fig. 6. Transmission electron micrographs of spore formation of *Mycosphaerella graminicola* isolate *IPO87016* in cv. Shafir. A, Section through a young pycnidium with conidiophores and conidia. Note septum (arrow) between immature conidium and conidiophore, lipids (arrowheads) and cell organelles, as well as the hygroscopic matrix supporting the conidia (asterisks), x6700. B, Longitudinal and transverse section through young conidia. Note first conidial septum (arrow) and the nucleus and other cell organelles in the upper segment of the longitudinal section, x8800. C, Detail of the septum in B, shows the wall-layering in the septum, the septal pore (arrowheads), and Woronin bodies, x35000.

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that eventually matured (Figs. 5B-D). The development of a young pycnidium into a conidium-producing mature pycnidium was completed in 2-3 days. Ripe pycnidia were invariably located under the stomata, usually one per substomatal cavity, in accordance with the linear arrangement of pycnidia in diseased leaves. Pycnidia were subglobose, with the ostioles confined by, and in the shape of, the stomatal openings, without any additional internal or external structures, such as the paraphyses, (Figs. 5B-D) observed in the hymenia of sexual fructifications. Microspores were not observed.

CONIDIOGENESIS AND SPORULATION (Figs. 6-7). Initially aseptate conidia were formed from conidiophores on the wall of the pycnidium (Figs. 5D and 6A). The wall of mature conidia consists of two layers, as also evident from the ultrastructure of the septae that divide the conidia in segments
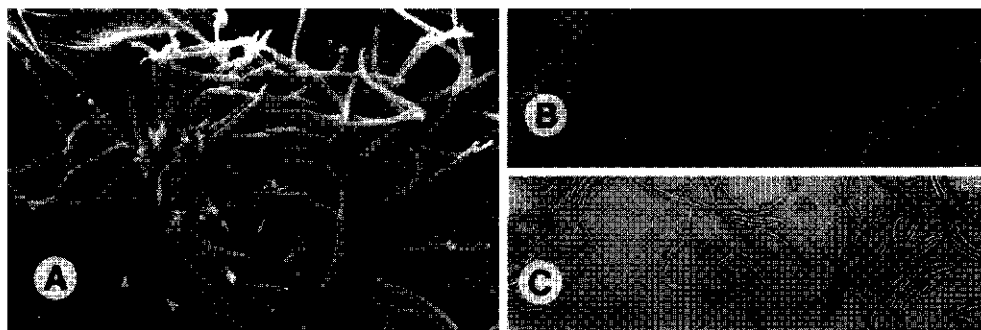
Fig. 7. Scanning electron and light micrographs of asexual conidium liberation in *Mycosphaerella graminicola*. A, Micrograph of a mature pycnidium at 12 dpi. Remnants of a conidial cirrhus after critical point drying preparation. Erosion tracks after the dissolution of epicuticular wax (arrowheads), caused by germ tubes and old mycelium on the leaf, x2413. **B and** C, Exuded conidia stained with DAPI and viewed with UV epifluorescent microscopy (**B**), and regular light microscopy (**C**), respectively, illustrating that each conidial cell is uninucleate, x1000.

(Figs. 6B, C). Conidia are exuded through the pycnidial ostiole in a cirrhus (Fig. 7A), due to the hygroscopic characteristics of the matrix in the pycnidium. Each cell of a mature septate conidium contains one nucleus (Figs. 6B and 7B,C).

MYCELIUM QUANTIFICATION. The development of antigen within the leaves was immediately detectable in extracts from inoculated plants, but the level of antigen did not change significantly on either cultivar until 6 dpi. Thereafter, the antigen level in cv. Shafir increased exponentially at a constant rate until the end of the experiment (14 dpi). The antigen level in extracts from cv. Kavkaz/K4500 1.6.a.4 remained at a constant low level throughout the experiment (Fig. 8). The assay was not detectably non-linear against log [antigen concentration] within the range of the standards provided by the kit.
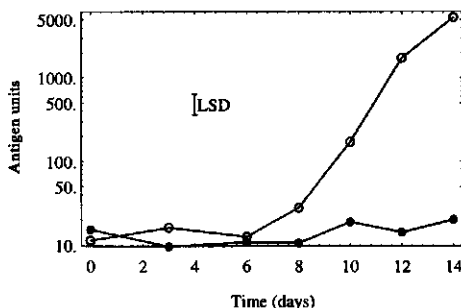
Fig. 8. Antigen quantification of *M. graminicola* isolate *IPO87016* with a proprietory kit based on ELISA (DuPont, Stevenage, UK) at different time intervals in compatible and incompatible interactions with the wheat cvs. Shafir (○) and Kavkaz/K4500 1.6.a.4 (●), respectively.

DISCUSSION

TOPOGRAPHIC STRUCTURES or chemical signals are necessary for germ tube orientation and penetration in many pathogens (51). Many rust species rely on a combination of chemotropism and thigmotropism for the production of appressoria on stomata, which are necessary for successful penetration of the host (2). Firm attachment of germ tubes to leaf surfaces is a prerequisite for oriented growth and appressorial induction, hence for pathogenicity, and is often visualized by erosion tracks after dissolution of host cuticular components such as waxes (90). Although we observed such tracks, germ tubes appeared to grow largely at random over the leaf surface, regularly crossing stomata without penetration. Hence, under our experimental conditions thigmotropic or chemotropic signal perception in *M. graminicola* did not seem to be of importance. The difference of mycelial content in leaves sampled at 12 or 24 hpi and those sampled at 48 hpi is additional evidence for this conclusion. In spite of the observed presence of hyphae in substomatal cavities at 12 hpi, only plants that were incubated for 48 h developed *M. graminicola* symptoms. This observation accords with data published by Shaw (117), though he observed also differences between a susceptible and a moderately susceptible cultivar, as well as effects of light on the infection frequency. An incubation period of 48 h might enhance the probability of penetration. The fungus apparently requires an incubation period of 48 h to reach the mesophyll cells, a prerequisite for further colonization. The

appressorium-like structures that were observed on stomata were not necessarily involved with successful penetration, and are therefore not considered to be appressoria in the true sense, *i.e.* organs necessary for penetration as in many rust species. They may be organs that help the fungus to attach to the plant surface rather than to penetrate the host, particularly because they were also observed on epidermal cells (16,136). Our experiments provide evidence only for stomatal penetration of *M. graminicola* and did not confirm reports of direct penetration (16,136).

DIFFERENCES BETWEEN responses on susceptible and resistant cultivars were already evident from 48 hpi and increased thereafter, particularly with respect to the form and number of chloroplasts and nuclei in the susceptible and intermediate responses of cv. Shafir to isolates *IPO87016* and *IPO235*, respectively. These effects also occurred without the presence of hyphae in the proximity of the mesophyll cells, particularly with the latter isolate. The condensation of chloroplasts could be an early response to soluble compounds excreted by the fungus, subsequently followed by intense swelling in cells at the verge of collapse. In addition to the chloroplast alterations, starch granules seemed to be released from the chloroplasts, which was not observed in the interaction with isolate *IPO87016*. The role of this phenomenon in unknown, but starch may be processed in an attempt to delimit further colonization. In our study the process of chloroplast condensation and degeneration was initially not at all linked with necro-

tization and did not significantly influence the efficiency of photosynthesis as measured by chlorophyll fluorescence until stages later than 10 dpi (M.J. Prins, C. Kliffen and G.H.J. Kema, *unpublished*). Such changes, therefore, might be symptomatic of disturbed sink-source relationship, and reflect the sensitivity of wheat mesophyll cells to stress factors, since they were also observed in wheat leaf tissues infected with wheat streak mosaic rymovirus and *Pyrenophora tritici-repentis* (39,72). The aforementioned alterations were not observed in incompatible responses where hyphae were only occasionally observed, without any visible detrimental effect on mesophyll cells.

THE STRICTLY intercellular growth of the fungus provides a clue for the elucidation of the pathogenesis of *M. graminicola* in wheat. It implies that communication between the plant and the fungus takes place in the apoplast, which creates perspectives for analysis of intercellular washing fluids, and for elucidation of resistance and virulence in this pathosystem (25). Similar approaches for the interaction of wheat with *P. tritici-repentis* revealed necrosis-inducing proteins, which appear to be pathogenicity factors (71). Our observations suggest the involvement of soluble toxic compounds in the wheat - *M. graminicola* pathosystem, since *i*) in compatible interactions, mesophyll cells were severely affected without the presence of mycelium in the vicinity, and *ii*) cell collapse occurred within a short span of time. However, cell collapse was not observed before 8 dpi, which might

indicate that a critical amount of fungal biomass or a specific physiological state is required to induce necrosis. Further TEM analysis of the interaction between *M. graminicola* isolate *IPO87016* and cvs. Shafir and Kavkaz/K4500 l.6.a.4 is in progress.

THE PATHOGENESIS of *M. graminicola* in wheat differs from that of other perthotrophic fungi on cereals such as *P. tritici-repentis* and *Rhynchosporium secalis* (71,72,73,77,78). These pathogens do not penetrate the stomatal aperture, but colonize the epidermal cells producing appressoria to penetrate the anticlinal and/or periclinal cell walls. Cell collapse, due to necrosis inducing phytotoxic compounds (43, 71), in these model systems occurs within a few days after inoculation. Hyphae of *R. secalis* are observed between mesophyll cells only at very late stages of pathogenesis (73), and resistance was associated with the production of papillae, which were not observed in the current experiments with *M. graminicola.*

PECTIN DEGRADING enzymes may play a role in the pathogenesis of *M. graminicola* in a susceptible host, that can be appropriately studied by using toluidine-blue which stains acid pectin. In contrast to the mesophyll cell walls of the water inoculated controls of cv. Shafir, mesophyll cell walls in the proximity of hyphae of isolate *IPO-87016* were hardly stained. However, papillae or any other cell wall strengthenings were not observed in conjunction with the occasional presence of *M. graminicola* isolate *IPO87016* between mesophyll cells of the resis-

tant cv. Kavkaz/K4500 1.6.a.4. The resistance mechanism, therefore, does not seem to rely on defence responses to fungal cell-wall degrading enzymes, but rather on the production of compounds that preclude fungal colonization, and hence pycnidium formation. In our experiments, pycnidium formation only occurred after massive cell collapse, which presumably provided the necessary release of nutrients for further colonization and eventual production of these asexual fruiting bodies in the substomatal cavities, which was already described in classical *M. graminicola* literature (4,12,32). The resistance mechanism of cv. Kavkaz/K4500 1.6.a.4, therefore, does not seem to be linked with arrested pycnidium formation as suggested by Cohen and Eyal (16).

MINIMAL INCREASE of fungal tissue, if any, in cv. Kavkaz/K4500 1.6.a.4 was also evident from the ELISA experiment. Significant differences between cvs. Shafir and Kavkaz/K4500 1.6.a.4 occurred at 8 dpi and later, thus after the first cell collapse, which initiated rapid colonization. The slight increase of fungal tissue observed microscopically in cv. Shafir during the 48 hpi - 8dpi interval, prior to the first cell collapse, was not detected by ELISA. Nevertheless, ELISA of the antigen of *M. graminicola* provides a quantitative measure of fungal proliferation within a leaf. In addition to the cytological information of the pathogenesis of *M. graminicola* in wheat, the antigen level seems likely to be proportional to the fungal biomass in the leaf, and not preferentially

produced during certain physiological states of fungal growth. The assay, therefore, may contribute considerably to the understanding of the interaction between *M. graminicola* and wheat, as was recently reported for the *Leptosphaeria maculans-Brassica napus* pathosystem (6), and offers prospects as a rapid alternative to evaluate inoculation experiments to characterize varying levels of resistance in the host and virulence in the pathogen.

THE ROLE of phytoalexins in disease resistance is evident in diverse pathosystems (44,130) and phytoalexin detoxification might be responsible for phytoalexin tolerance and as such indispensable for the expression of virulence (110,130). Hence, virulence in isolate *IPO87016* to cv. Shafir might be controlled by detoxification of phytoalexins, which enables colonization of the mesophyll tissue and eventually the production of sufficient toxic compounds to kill host cells. In that case, pycnidium production might be considered as a strictly autonomous process following cell collapse. Kema et al. (61,63) suggested that necrosis induction and pycnidia production were under different genetic control since they observed a poor correlation between these parameters, which is exemplified in the interaction of cv. Shafir with isolate *IPO235*. Such a response might be explained by incomplete inactivation of triggered antifungal compounds, which allows restricted colonization and eventually toxin production, but cell collapse evidently does not initiate mycelial proliferation, resulting in limited, local pycnidium formation. Analysis of
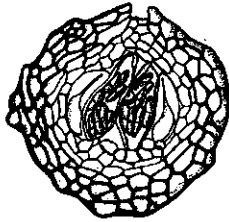
intercellular washing fluids appears to be essential to resolve those aspects of host-pathogen interactions in the wheat - *M. graminicola* pathosystem.

GENERAL DISCUSSION

ANALYSIS OF pathosystems comprises many aspects, such as the biology and the host range of the pathogen. A further specification of the host range of a pathogen is to determine whether cultivars respond more or less similar to pathogen isolates or not. This phenomenon is called specificity. The order in which such aspects are addressed in research is most important for the perception of a pathosystem. In the cereal rusts it became clear at an early stage that physiological races existed. Later on the role of sex in the generation of genetic variation in the pathogen was evidenced. These findings were of great importance for the establishment of the gene-for-gene concept that was developed by Flor (36,37), which led to recognition of similar mechanisms in other pathosystems (123). For the wheat-*M. graminicola* pathosystem this process has been much less clear. Early publications on *M. graminicola* particularly dealt with the biology and the host range of the fungus (24,50,136). Specificity, following the gene-for-gene concept, was suggested by Eyal et al. (31) in 1973. Since then, unfortunately, scepticism dominated and no firm foundation for an appropriate evaluation of specificity in the wheat - *M. graminicola* pathosystem was established by additional research. This strongly influenced the perception of this pathosystem. In addition to that, the paradigm of boom-and-bust cycles, as occurring in the wheat-rust pathosystems, dominated the perception of the epidemiology of wheat diseases, as expressed recently by Johnson (56).

Since such events did not appear to happen in the wheat-*M. graminicola* pathosystem, resistance and pathogenicity were usually implicitly, but sometimes explicitly designated as quantitative characters. Recently, Parlevliet (94) categorized *M. graminicola* among diseases where a few or no races are known, and either a few specific resistance genes are recognized or genes have been effective since their introduction. Apart from the fact whether it makes sense to designate races in *M. graminicola*, this opinion does not accord with the complexity of the wheat-*M. graminicola* pathosystem, and is an over-simplification of reality. Moreover, Van Ginkel and Scharen (134) speculated that the observation of Eyal et al. (31) was probably due to host species specialization. In other words, Eyal et al. (31) did not realize that *M. graminicola* isolates from durum wheat affected durum wheat cultivars, and isolates from bread wheat did not. Reversely, isolates from bread wheat appeared to be specialized on that species. In chapter one, this idea is firmly evidenced among isolates originating from the Mediterranean area. Only a minority of isolates were able to produce considerable amounts of pycnidia in cultivars of both species, sometimes irrespective of high necrosis values. Interestingly, such an adaptation was also observed in leaf rust from the same region (3,34). Recently, a screening of 117 isolates originating from durum wheat and bread wheat originating from Mediterranean countries clearly confirmed adaptation in *M. graminicola* to either of the two wheat species, even among isolates from the same location (M.R.

Simón and G.H.J. Kema, *unpublished*). Analysis of the nuclear and mtDNA of these isolates revealed that there is probably limited, if any, genetical exchange among the two forms. Interestingly, a previously unobserved mtDNA RFLP pattern was only observed among the durum wheat adapted isolates (G. Trautweiler, B.A. McDonald, and G.H.J. Kema, *unpublished*).

HOWEVER, THE suggestion of Eyal et al. (31) was prematurely abandoned (56, 134), which was partly due to the fundamental lack of knowledge with respect to the pathogenesis of *M. graminicola* in wheat. In chapter five it is described that necrosis does occur beyond the presence of mycelium. Hence, it leaves a possibility that necrosis may be part of a resistance response, which makes a virulence analysis based on this parameter less reliable.

Another legitimate requirement for claiming specificity is to study the relation between data obtained under controlled conditions and those obtained under field conditions. Such an experiment could have been carried out much earlier if one had realized that pathogenic differences among *M. graminicola* isolates were of importance. The fact that such experiments were not or inappropriately conducted, by using natural populations, isolate mixtures or a single isolate, is a testimony for the lack of conclusiveness with respect to specificity in *M. graminicola*. The results of chapter three are therefore of importance. For the first time such comparisons clearly demonstrated specificity in the wheat-

M. graminicola pathosystem under field conditions. These experiments need to be repeated and confirmed with different host and pathogen genotypes. One cannot, at least not in the current field of science, derive conclusions from strong but limited evidence.

IN THIS thesis host species and host cultivar specialization in *M. graminicola* are unequivocally demonstrated. Chapter two mainly described statistical procedures to provide a firm mathematical base for specificity. This was necessary to refute the belief that quantitativeness is all what matters in the wheat-*M. graminicola* pathosystem. Unfortunately, such data came far too late. An earlier consensus on specificity in *M. graminicola* would have contributed to the correct perception of this pathosystem in a much earlier stage.

Sanderson's findings with respect to the ascogenous state of the pathogen (108,109) would have been placed in the correct perspective, *i.e.* the distribution of a population with different qualitative and quantitative virulence characters. Hence, the possibility of an 'apparent' quantitativeness of the pathosystem. This latter suggestion agrees with Parlevliet's question (93) whether horizontal resistance can be recognized in the presence of vertical resistance if plants are exposed to a mixture of pathogen races. Population dynamics research using molecular markers by McDonald and coworkers (81,82,83, 84,85) demonstrated the vast genetic variation for such markers in *M. graminicola* populations. Clonal lineages were hardly found (85). This result suggests an important role of the sexual stage in the epidemiology of the

pathogen. The results described in chapter four clarified the mating system in *M. graminicola* and the occurrence of multiple generative cycles. These findings are perfectly in line with the data of McDonald and coworkers. A major question remains: does genetic variation for molecular markers imply a similar magnitude of variation for virulence characteristics? This thesis shows that differences among isolates, even from the same location, can be very large. Hence, the expectation is that the abovementioned question can be answered positively, but the final answer has to come from new experiments.

HAVING REVIEWED the major role of the sexual cycle in the epidemiology of *M. graminicola*, a part of the epidemiological work of Shaw and Royle (104,118) should be discussed. Their emphasis on splash dispersal of pycnidiospores as the major vehicle for disease progress might not be correct. The entanglement of the dispersal of sexual and asexual propagules by similar weather conditions was not envisaged because it was not known that multiple generative cycles occur, probably throughout the season. As a matter of fact, weather conditions for ascospore dispersal appear to occur much more frequently than 'splashy rains' for dispersal of pycnidiospores. An important field of research therefore is to identify the relative contribution of asexual and sexual propagules to disease progress. Using RFLP markers, McDonald et al (85) suggested that the role of ascospores will dominate over pycnidiospores. There is no difference in opinion about the major role of

ascospores in the establishment of founder populations (104,109,117). In many countries, including The Netherlands, ascospore flights in autumn are responsible for initial infections of young wheat crops. Hence, an obvious control measure would be to prevent such ascospore flights. Sanitation in time or space is the first option. Late planting of new wheat crops usually decreased the incidence of the pathogen (8,19). Control of *M. graminicola* on the wheat stubble would probably be much more effective for the control of septoria tritici blotch. This might be achieved by applying fungicides, which had e.g. dramatic effects on the ascospore flights of *Leptosphaeria maculans* from canola stubbles (97). Alternatively, biological control agents can be applied in the same period, either in conjunction with or apart from such treatments, to delimit the formation of ascocarps (69,98).

FINALLY, THE molecular genetic analysis of *M. graminicola* is an important field of research to be explored. Genetic analysis of virulence in the pathogen will elucidate whether the gene-for-gene concept applies to the wheat - *M. graminicola* pathosystem. Breeders will benefit from this type of research since it will enable to study the dynamics of (a)virulence genes in pathogen populations, and to compare the frequencies of such genes in different pathogen populations. Isolation of mating type genes will enable their detection in pathogen populations, hence the expected genetic variation in such a population. This is of particular interest to the Mediter-

ranean area, since it is at present unknown whether the 'durum type' and the 'bread wheat type' of *M. graminicola* do exchange genetic information. Preliminary results (G. Trautweiler, B.A. McDonald, and G.H. J. Kema, *unpublished*) indicate that this is probably not the case in that particular area. Manipulation with mating type genes may also provide a fundamental method to prevent ascospore formation, which would be an extremely effective control measure. The 'barberry eradication campaign' in the USA in the early 20-ties has shown which effects such preventive measures may have on the composition of the pathogen population, and as such on the success of breeding efforts (42,86).

BREEDING FOR resistance is obviously another important approach to control septoria tritici leaf blotch (131). However, the effectiveness of breeding for resistance is severely hampered by insufficient knowledge of the genetic variation in the pathogen population. Moreover, reports on the mode of inheritance of resistance to *M. graminicola* have been inconsistent with respect to the numbers and the effects of the genes involved (21,57,88,132, 133). This is not surprising since different approaches and disease parameters were used to screen and evaluate the segregating populations. Exposing segregating host plant popu-

lations to natural infection, most probably due to a segregating pathogen population, is certainly an inappropriate method to study the genetics of resistance to *M. graminicola*. One has to realize that the genetic variation for virulence and resistance in pathogen and host, respectively, may swing between factors with relatively large and relatively small effects. An approach which meets at least partly the aforementioned objections, is to use single isolates of the pathogen, which enables selection for non-specific resistance in the host (93,94). A major question is: which isolate should be applied? This question cannot be answered in an easy way since one should take into account the vast genetic variation in the natural population. One approach could be the method described in chapter two. Collect a reliable sample of the natural population and determine the σ^2_p of each isolate on a range of cultivars. Isolates with a high σ^2_p may be considered to carry a relatively high number of specific factors. The $P_{overall}$ should be used to check whether isolates with a low σ^2_p are either generally avirulent or virulent. Hence, two possibilities arise: as a screening isolate, use one with a high σ^2_p or one with a low σ^2_p but a high $P_{overall}$. Such an approach would at least contribute to a more conscious breeding against septoria tritici leaf blotch with the emphasis on non-specific resistance.

SUMMARY

THE RESEARCH described in this thesis is dealing with *i*) the extent of genetic variation, *ii*) the generation of genetic variation and *iii*) the pathogenesis of *Mycosphaerella graminicola*, a fungal pathogen causing septoria tritici leaf blotch in wheat. The disease is characterized by necrotic blotches that contain the fruiting bodies. These can be either asexual pycnidia or sexual pseudothecia, carrying the pycnidiospores and ascospores, respectively. Pycnidiospores are splash-dispersed and ascospores are air-borne. Both contribute to disease progress, though their relative contributions are unknown. Septoria tritici leaf blotch is of increasing importance in regions with a temperate climate. Increased importance in Europe is most probably related with the change of cultural practices and with the improvement of the control of other foliar pathogens.

The first three chapters of this thesis describe genetic variation for virulence among 80 isolates of the fungus, which was determined by testing wheat cultivars under controlled conditions and in the field. Chapter one deals with genetic variation for virulence in 63 isolates that originated from 13 countries. The experiments were performed in the seedling stage according to a partially balanced incomplete block design with four replications over time, and included both bread wheat and durum wheat cultivars as well as isolates secured from these species. Two disease parameters, the presence of necrosis (N) and pycnidia (P)

estimated as square percentages on primary leaves, were used to measure disease severity. Genetic variation for virulence in the pathogen isolates and genetic variation for resistance in the host cultivars were estimated by analyses of covariance. The significance of cultivar x isolate interactions in all experiments and for each disease parameter suggests differential interaction, which may be attributed to a gene-for-gene interaction between resistance and virulence loci in host and pathogen, respectively. An agglomerative hierarchical clustering procedure, which used one *df* components of interaction between isolates and cultivars as a proximity measure, was employed to study the similarity between isolates and cultivars. Discrepancies between N and P resulted in non-identical clusters of isolates and cultivars when considering these parameters separately, which suggests that N and P are under different genetical control. Moreover, isolates appeared to be adapted to either bread wheat or durum wheat, which was particularly evident when considering P. It is proposed, therefore, to designate two varieties in *M. graminicola*, which refer to the host species specialization in this pathogen.

Chapter two describes various statistical procedures to analyse host-pathogen interaction, and verification experiments to validate the proposed methodologies. Non-parametric and parametric statistical procedures were employed to analyze six data sets comprising 80 pathogen isolates and 47 host cultivars to detect the presence

and estimate the relevance of interaction in the wheat-*M. graminicola* pathosystem. Each data set was confined to either responses of bread wheat to bread wheat-derived isolates, or of durum wheat to durum wheat-derived isolates, and to each of the two disease parameters. Four data sets were employed for explorative parametric statistical analyses, involving a procedure using the size of the overall variances for cultivars and isolates in tables of effects to estimate the relative proportions of specific factors for resistance and virulence in host and pathogen genotypes, respectively. Subsets that comprised cultivars and isolates with either high or low variances, were selected from the data matrices and were subjected to analyses of covariance. Those that included entries with high variances revealed interaction mean squares that explained approximately 25% of the total variance, which was considerably higher than in the complete data matrices. The results indicate considerable genetic variation for specific resistance and virulence factors in host and pathogen, respectively, and hence for the effectiveness of the procedure. Analysis of subsets that were confined to entries with low variances resulted in interaction mean squares that contributed little to the total variance, which is an indication for the absence of differential responses, that might be due to either susceptible or resistant responses to all applied pathogen isolates. The statistical procedure was validated by performing an additional selection experiment, which confirmed the hypothesis that a large overall variance is indicative of specific factors

for virulence or resistance. It also indicated that a low overall variance is not necessarily indicative of nonspecific resistance. These conclusions were strengthened by non-parametric statistical procedures that showed significant interactions between pathogen isolates and host cultivars. Hence, interactions in parametric statistical analyses were not due to stretch. Similar results were obtained for both disease parameters.

Chapter three describes comparative seedling-adult plant experiments. These were performed to investigate the occurrence of host-cultivar interactions under field conditions, and as such also enabled the determination of the predictive value of responses measured in the seedling stage for responses in the adult plant stage. The seedling data were analyzed using cluster analyses to perform an unprejudiced selection of isolates with significantly different virulence characteristics for adult plant tests. These were conducted in two field experiments in 1992 and 1995. Inoculations were conducted after flowering to avoid effects of earliness and tallness, hence the experiments were intended as monocyclic tests for virulence differences between the isolates. Significant cultivar x isolate interactions were observed for both disease parameters in each experiment. This result confirmed conclusions derived from seedling experiments and supports the hypothesis of specificity in the wheat - *M. graminicola* relationship. Correlations between seedling and adult plant data were significant for only one of the three applied isolates. Hence, evaluation of resistance and

virulence might require seedling as well as adult plant tests.

Chapter four describes the mating system of *M. graminicola*, a procedure to cross isolates, and the genetic analyses of progenies from such crosses. Mono-spore isolates derived from ascospores that were considered to originate from one ascus were studied by the polymerase chain reaction (PCR) with 33 RAPD primers. Three of those primers were used to study segregation of RAPD markers in three randomly selected ascospore progenies of 54 isolates. Mendelian segregation of RAPD markers was observed. In one progeny, Southern blot homology studies indicated that two identical 800 bp RAPD markers were on different loci, probably as a result of translocation. The results strongly point at a bipolar heterothallic mating system in *M. graminicola*, which has important epidemiological consequences, and provides an explanation for the vast genetic variation in this pathogen.

Chapter five describes the pathogenesis of *M. graminicola* in the susceptible cultivar Shafir, and the resistant cultivar Kavkaz/K4500 l.6.a.4. Experiments were designed as time-sequence studies in two replications with sampling dates at 12, 24 and 48 hours post inoculation (hpi), and 4, 8, 10, 12, 14 and 16 days post inoculation (dpi). Samples were studied by light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The germination frequency of *M. graminicola* spores was high in both compatible (Shafir) and incompatible interactions (Kavkaz/K4500 l.6.a.4),

though the infection frequency was low. Infection was strictly stomatal but appeared to be a random process, since many germ tubes crossed stomata without penetrating them. Some germ tubes formed branched structures close to or on top of stomata. These structures were small compared to the size of stomata, were formed irregularly and were not significantly correlated with successful penetrations of the host. Multiple penetrations of stomata occurred regularly. Hyphae were already observed in the substomatal cavities at 12 hpi. In the compatible response, colonization was fairly limited until 8 dpi. Hyphae grew intercellularly and in close contact with the mesophyll cells. During the 10-12 dpi interval excessive cell death occurred, which induced further colonization that eventually resulted in pycnidia formation in the substomatal cavities. Initial and further colonization had a strong and negative effect on the numbers and sizes of the chloroplasts in the compatible interaction, though macroscopically leaves remained green until ca. 10 dpi. The resistant response was primarily characterized by a very limited colonization, often restricted closely to the substomatal cavity. This was also evidenced in a separate experiment that was performed to quantify a *M. graminicola* antigen in both cultivars at the aforementioned intervals using the DuPont double antibody sandwich ELISA assay for *M. graminicola*. Data analysis suggested that the mycelial quantities in both cultivars remained similar until eight days after inoculation. After that, the mycelial quantity developed exponentially in cv. Shafir

but did not significantly increase in cv. Kavkaz/K4500 l.6.a.4. Therefore, the resistance mechanism in wheat against *M. graminicola* appears to be based on inhibition of fungal proliferation.

Finally, in the general discussion, the result of the research described in the preceding chapters is put in a wider framework. Suggestions for additional research, necessary for an improved understanding and control of the wheat - *M. graminicola* pathosystem, are put forward.

ET IN dit proefschrift beschreven onderzoek behandelt *i*) de mate van genetische variatie, *ii*) de vorming van genetische variatie en *iii*) de pathogenese van *Mycosphaerella graminicola*, een pathogene schimmel die septoria bladvlekkenziekte in tarwe veroorzaakt. De ziekte wordt gekenmerkt door necrotische vlekken die de vruchtlichamen bevatten. Deze vruchtlichamen kunnen zowel ongeslachtelijke pycnidiën, als geslachtelijke pseudotheciën zijn, die resp. de pycnidiosporen en ascosporen bevatten. Pycnidiosporen worden door regendruppels verspreid en ascosporen door de wind. Beide soorten sporen dragen bij aan de toename van de ziekte in het gewas, maar hun relatieve bijdrage is onbekend. Septoria tritici bladvlekkenziekte is een toenemend probleem in de gematigde klimaatgebieden. In Europa is dit waarschijnlijk deels toe te schrijven aan gewijzigde teeltomstandigheden en aan de verbeterde bestrijding van andere ziekten.

De eerste drie hoofdstukken van dit proefschrift beschrijven de genetische variatie voor virulentie in 80 isolaten van de schimmel. Deze werd bepaald door het toetsen van tarwerassen met de isolaten onder geconditioneerde omstandigheden en in het veld.

Hoofdstuk 1 behandelt de genetische variatie voor virulentie in 63 isolaten afkomstig uit dertien landen. De proeven werden uitgevoerd op kiemplanten volgens een gedeeltelijk gebalanceerd onvolledig blokken ontwerp met vier herhalingen in de tijd. In alle proeven waren rassen van zowel brood- als durumtarwe betrokken, alsmede isolaten van deze soorten. De ernst van de ziekteverschijnselen werd geschat met behulp van twee ziekteparameters: het percentage necrose (N) en het percentage pycnidiënbezetting (P) van het eerste blad. De genetische variatie voor virulentie in de schimmelisolaten en de genetische variatie voor resistentie in de tarwerassen werd beschreven na covariantie-analyses. De significantie van de ras x isolaat interacties voor beide ziekteparameters in alle experimenten suggereert het optreden van een gen-om-gen interactie tussen resistentie en virulentie loci in respectievelijk de waardplant en het pathogeen. De overeenkomst tussen rassen en isolaten werd bestudeerd met behulp van een statistische techniek (clusteranalyse) waarbij voor groepsvorming minimale interacties tussen rassen en isolaten werden gehanteerd. Bij toepassing van deze methoden op datasets van beide ziekteparameters traden verschillen op in samenstelling van de gevormde groepen, hetgeen suggereert dat de twee ziekteparameters genetisch verschillend worden gereguleerd. Bovendien bleken isolaten van de schimmel aanpassing te vertonen voor broodtarwe òf durumtarwe. Dit was vooral duidelijk bij beschouwing van P. Daarom is, in overeenkomst met deze aanpassing, voorgesteld om isolaten van de schimmel in te delen in twee variëteiten.

Hoofdstuk twee beschrijft verschil-

lende statistische technieken om waard-pathogeen interacties te analyseren en verificatie-experimenten om de voorgestelde methoden te toetsen. Verdelingsvrije en verdelingsgebonden statistische procedures werden gebruikt om in zes datasets, die 80 schimmelisolaten en 47 waardplantrassen omvatten, interacties op te sporen en de relevantie ervan in het tarwe-*M. graminicola* pathosysteem te bepalen. Elke dataset beperkte zich tot *N* òf *P* en bevatte òf gegevens met betrekking tot brood-tarwerassen en broodtarwe-isolaten òf durumtarwerassen en durumtarwe-isolaten. Vier datasets werden gebruikt om een statistische techniek te ontwikkelen die gebruik maakt van de varianties voor rassen over alle isolaten, en voor isolaten over alle rassen in effecttabellen. Hiermee kan de mate van specificiteit van de genetische factoren in rassen en isolaten worden geschat. Kleine subsets die enerzijds rassen en isolaten met hoge en anderzijds met lage varianties omvatten, werden onderzocht met behulp van covariantie-analysen. Hieruit bleek dat de bijdrage van de interactiekwadraatsommen aan de totale variantie in datasets met rassen en isolaten met hoge varianties aanzienlijk hoger was (≈25%) dan in de volledige datasets. De resultaten wijzen op een aanzienlijke genetische variatie voor specifieke resistentie- en virulentiefactoren, alsmede op de effectiviteit van de procedure om deze op te sporen. Uit analyses van kleine subsets die rassen en isolaten met lage varianties omvatten bleek dat hier de bijdrage van de interactiekwadraatsommen aan de totale variantie zeer

klein was. Hieruit kan de afwezigheid van ras x isolaat interacties worden afgeleid, veroorzaakt door een algemeen hoog of laag resistentieniveau in de getoetste rassen. De procedure werd geverifieerd in een selectie-experiment. Hierin kwam naar voren dat een grote variantie in de effecten tabel inderdaad een aanwijzing kan zijn voor de aanwezigheid van specifieke factoren. Kleine varianties zijn echter niet noodzakelijkerwijs een aanwijzing voor de afwezigheid van zulke factoren. Het beschrijven van 'horizontale' resistentie kan dus niet worden gebaseerd op deze methode. Tenslotte bleken de resultaten van een verdelingsvrije statistische methode alle conclusies met betrekking tot specificiteit te onderschrijven, wat aantoont dat interacties in verdelingsgebonden analyses niet het gevolg waren van de gebruikte procentuele waarnemingsschaal.

Hoofdstuk drie beschrijft vergelijkende kiemplant-volwassen plant experimenten. Deze werden uitgevoerd om het optreden van waardplant-isolaat interacties onder veldomstandigheden te onderzoeken, om een inzicht te verschaffen in de voorspellende waarde van de reacties van kiemplanten voor de reactie van volwassen planten. De kiemplantgegevens werden geanalyseerd met behulp van een clusteranalyse, waarna drie isolaten met significant verschillende virulentiekarakteristieken werden geselecteerd voor toetsing onder veldomstandigheden. Deze experimenten werden in 1992 en 1995 uitgevoerd. De planten werden geïnoculeerd na de bloei om raseffecten

met betrekking tot lengte en vroegheid te minimaliseren. Deze proeven kunnen worden gezien als mono-cyclische testen voor virulentie-verschillen tussen isolaten van de schimmel. Significante ras x isolaat interacties werden voor beide ziekteparameters in beide experi-menten waargenomen. Dit bevestigt conclusies met betrekking tot specificiteit in de relatie tussen tarwe en *M. graminicola*, die werden getrokken op basis van kiemplant-experimenten. Correlaties tussen kiem-plantgegevens en volwassen-plant-gegevens waren daarentegen slechts significant voor één van de drie toegepaste isolaten. Derhalve lijkt de evaluatie van resistentie en virulentie gewenst in zowel kiemplant- als volwassen plant-experimenten.

Hoofdstuk vier beschrijft het geslachtelijk systeem in *M. graminicola*. Dit kon worden vastgesteld, na de ontwikkeling van een procedure om isolaten van de schimmel te kruisen, en op grond van genetische analyse van nakomelingschappen van zulke krui-singen. Monospore-isolaten afkomstig van ascosporen, waarvan werd verondersteld dat zij uit dezelfde ascus kwamen, werden met behulp van de polymerase ketting reactie onderzocht met 33 RAPD (randomly amplified polymorphic DNA) primers. Drie van deze primers werden gebruikt om de uitsplitsing van RAPD merkers in drie nakomelingschappen, bestaande uit 54 willekeurig gekozen isolaten, te onderzoeken. De waargenomen uit-splitsingen van de RAPD merkers waren overeenkomstig de wetten van Mendel. In één nakomelingschap werd de homologie van twee RAPD merkers

van identieke lengte (800 bp) vastgesteld met behulp van 'Southern blotting'. Verder bleek dat deze merkers waarschijnlijk als gevolg van translocatie op verschillende plaatsen in het genoom gelokaliseerd waren. De resultaten geven een zeer sterke aan-wijzing voor de aanwezigheid van een bipolair heterothallisch geslachtelijk systeem in *M. graminicola*, hetgeen epidemiologische consequenties heeft en de grote genetische variatie in dit pathogeen verklaart.

Hoofdstuk vijf beschrijft de patho-genese van *M. graminicola* in het vatbare tarweras Shafir en in het resistente tarweras Kavkaz/K4500 I.6.a.4. De proeven werden uitgevoerd in twee herhalingen. Monsters werden genomen op 12, 24 en 48 uur na inoculatie, en op 4, 8, 10, 12, 14 en 16 dagen na inoculatie. De monsters werden bestudeerd met behulp van lichtmicroscopische (LM) en electro-nenmicroscopische technieken (SEM en TEM). De kiemingsfrequentie van *M. graminicola* sporen was erg hoog in zowel de compatibele (Shafir) als de incompatibele (Kavkaz/K4500 I.6.a.4) interactie. De penetratie frequentie was daarentegen laag. Infectie vond uit-sluitend via de huidmondjes plaats, doch leek verder volstrekt willekeurig omdat vele kiembuizen over huid-mondjes heen groeiden zonder deze te penetreren. Sommige kiembuizen ont-wikkelden vertakte structuren dichtbij of op de huidmondjes. Ze waren klein vergeleken met de grootte van de huidmondjes en waren niet significant gecorreleerd met geslaagde penetra-ties. Bovendien werd meervoudige penetratie van hetzelfde huidmondje regelmatig waargenomen. Twaalf uur

na inoculatie werd reeds schimmel-weefsel in de substomatale holte waargenomen. Echter, tot ca. acht dagen na inoculatie was de kolonisatie in het vatbare ras zeer beperkt. De schimmeldraden groeiden intercellu-lair en in nauw contact met de celwanden van het mesophyl. In de periode van 10-12 dagen na inoculatie trad massale celsterfte op, hetgeen de ontwikkeling van het schimmelweefsel sterk stimuleerde en uiteindelijk resulteerde in de vorming van de pycnidiën in de substomatale holten. Kolonisatie had een groot en negatief effect op het aantal en op de grootte van de bladgroenkorrels in de compatibele interactie. De bladeren bleven echter groen tot ongeveer 10 dagen na inoculatie. De resistente reactie werd voornamelijk gekarak-teriseerd door een zeer geringe kolonisatie, vaak slechts beperkt tot (delen van) de substomatale holte. Dit resultaat werd bevestigd in een extra proef waarin met behulp van ELISA de hoeveelheid antigeen tegen *M. graminicola* in beide tarwerassen werd gekwantificeerd. Tot ca. acht dagen na inoculatie bleef de hoeveelheid schimmelweefsel in beide rassen gelijk. Nadien nam de hoeveelheid schimmel-weefsel in het vatbare ras Shafir exponentieel toe terwijl zij in het resistente ras Kavkaz/K4500 1.6.a.4 gelijk bleef. Het resistentiemechanisme in tarwe tegen *M. graminicola* lijkt dan ook voornamelijk gebaseerd te zijn op remming van de schimmelgroei.

Tenslotte wordt in een algemene discussie het onderzoek, beschreven in dit proefschrift, in een breder kader geplaatst. Daartoe behoort een bespre-king van suggesties voor onderzoek dat noodzakelijk is voor een beter begrip en dus ook een betere hantering van het tarwe - *M. graminicola* patho-systeem.

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*The indicated publications are the basis of this thesis

CURRICULUM VITAE

Gerrit Haatje Jan Kema werd geboren op 21 december 1957 te Rotterdam en groeide op in een gezin van vijf kinderen te Barendrecht (ZH). Hij bezocht van 1970 tot 1975 de Christelijke Prinses Juliana Scholengemeenschap voor MAVO te Dordrecht, en vervolgde zijn opleiding in dezelfde plaats aan het toenmalig opleidingscentrum van het Koninklijk Nederlands Landbouw Comité (KNLC), waar hij twee jaar (1975-1976) middelbaar en, na een voorbereidend jaar, vijf jaar hoger agrarisch onderwijs genoot. Hij studeerde in 1982 af in de richting Plantenteelt met een scriptie over het gebruik van wilde graansoorten in de resistentieveredeling van tarwe tegen roestziekten, waarvoor hij de KNLC scriptieprijs ontving. Tijdens de landbouwkundige opleiding te Dordrecht liep hij stage bij diverse landbouwbedrijven in binnen- en buitenland en bij het DLO-Instituut voor Planteziektenkundig Onderzoek (IPO-DLO) te Wageningen.

In 1982 werd hij door het Nederlands Graan Centrum bij het IPO-DLO aangesteld in een tijdelijke functie als onderzoeksmedewerker. In het kader hiervan inventariseerde hij tot 1984 de aanwezigheid van resistentiegenen tegen gele roest in tarwerassen en -lijnen van Nederlandse tarweveredelingsbedrijven en veredelingsinstituten. In 1984 werd hij bij het IPO-DLO aangesteld als onderzoeksmedewerker in een deeltijdfunctie (32 uur). Tevens begon hij de studie Plantenveredeling aan de Landbouwuniversiteit te Wageningen, die in 1991 werd afgesloten met afstudeervakken in Plantenveredeling en Fytopathologie (gecombineerd) en Plantentaxonomie. Vanaf 1987 organiseerde hij samen met collega's cursussen in diverse ontwikkelingslanden in het kader van een onderzoeksprojekt met betrekking tot bladvlekkenziekte van tarwe. Sinds 1991 is hij als wetenschappelijk onderzoeker verbonden aan het IPO-DLO en werkt hij aan septoria bladvlekkenziekte en gele roest van tarwe. Gert Kema is getrouwd en vader van zes kinderen.