Molecular and phenotypic analyses of pathogenicity, aggressiveness, mycotoxin production, and colonization in the wheat-*Gibberella zeae* pathosystem

Christian Joseph R. Cumagun

VERLAG GRAUER · Beuren · Stuttgart · 2004

Cumagun, Christian Joseph R.:
Molecular and phenotypic analyses of pathogenicity, aggressiveness, mycotoxin production, and colonization in the wheat-*Gibberella zeae* pathosystem.
VERLAG GRAUER, Beuren, Stuttgart, 2004.

ISBN 3-86186-453-3

D 100
Dissertation University of Hohenheim, State Plant Breeding Institute, 2004.

Dissertation thesis of the University of Hohenheim, Faculty of Agricultural Sciences, for the acquisition of the degree of a 'Doktor der Agrarwissenschaften (Dr. sc. agr.)' (Ph.D. in Agricultural Sciences).

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2. Co-reviewer:	Prof. Dr. H. Buchenauer
3. Co-examiner:	Prof. Dr. R. Blaich
4. Assistant Dean and Head of the Committee:	Prof. Dr. A. Valle Zárate
Date of oral examination:	25. March 2004

Printed with support from the German Academic Exchange Service (DAAD).

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Printed in Germany.
Druck: F. u. T. Müllerbader GmbH
Forststr. 18, 70794 Filderstadt, Germany

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To Johann Siegfried, our third child
who was born two days before
my Ph.D. exam date

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[2]Cumagun, C.J.R., Parzies, H.K., and Miedaner, T. 2003. Genetic variation and segregation of DNA polymorphisms in *Gibberella zeae* detected with AFLP and RAPD markers (unpublished).

[3]Cumagun, C.J.R., and Miedaner, T. 2004. Segregation for aggressiveness and deoxynivalenol production of a population of *Gibberella zeae* causing head blight of wheat. Eur. J. Plant Pathol. (in press).

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Abbreviations

ADON	acetyldeoxynivalenol
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BAC	bacterial artificial chromosomes
bp	base pair(s)
CIM	composite interval mapping
cM	centiMorgan
CTAB	hexadecyltrimethylammonium bromide
dNTPs	deoxynucleotide triphosphates
DNA	deoxyribonucleic acid
DON	deoxynivalenol
ELISA	enzyme-linked immunosorbent assay
ERG	ergosterol
EST	expressed sequence tag
ExAg	exoantigen
FHB	Fusarium head blight
GUS	ß-D-glucuronidase
HOH	Hohenheim
LOD	logarithm of odds
MAPKs	mitogen-activated protein kinases
MAT	mating type
nit	nitrate nonutilizing
NIV	nivalenol
OD	optical density
OLI	Oberer Lindenhof
OPT	Operon Technologies
PCR	polymerase chain reaction
PDA	potato dextrose agar
PATH1	pathogenicity (qualitative gene)
PER1	perithecial production
PIG1	red pigment production
PTA	plate trapped antigen
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
SDW	sterile distilled water
SIM	simple interval mapping
SNA	synthetic nutrient-poor mineral agar
TAE	tris-acetate-EDTA
TE	tris-EDTA
TES	tris-EDTA-SDS
TFPGA	tools for population genetic analysis
TOX1	trichothecene toxin amount
TRI5	trichodiene synthase
UBC	University of British Columbia
UPGMA	unweighted pair-group method
VCGs	vegetative compatibility group

1 General Introduction

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is a filamentous ascomycete that infects diverse plant species and those of economic importance include maize and small grains such as wheat, barley, rye, triticale, and rice (Cook, 1981). This major causal agent of scab or Fusarium head blight (FHB) of wheat, reduces grain yield and quality and contaminates cereal grains with trichothecene and estrogenic mycotoxins that render the harvest non-marketable and pose health risks in feed and food products (Marasas et al., 1984, Dubin et al., 1997). Disease symptom is characterized by a water- soaked appearance of the spikelets which eventually become straw-coloured. Where conditions are highly favorable, pink-red mycelium and conidia developed on the spikelets and infection spreads through the entire head. Kernels ultimately become discoloured, shrivelled and chalky white in appearance hence the name "scab" (Wiese, 1987). Conidia (asexual) and ascospores (sexual) are produced which are both important in infecting wheat heads in the field (Stack, 1989). From 1991 to 1997 US farmers lost $ 2,600 million due to severe head blight epidemics and subsequent mycotoxin contamination of wheat and barley (Windels, 2000) and an additional $ 870 million from 1998 to 2000 (Nganje et al. 2001).

Aside from *G. zeae*, FHB is caused by several other species and the most common are *G. avenaceae* R. J. Cook (anamorph: *F. avenaceum* Corda ex. Fr.), *F. culmorum* (Smith) Sacc., *F. poae* (Peck) Wollenw., and *Monographella nivalis* (Schnaffit) E. Müller (anamorph: *Microdochium nivale*). *G. zeae* is normally prevalent in continental regions in Asia, North and South America and Europe whereas in temperate or maritime regions, *F. culmorum* is most common (Parry et al., 1995). There has been subsequent epidemic outbreaks of FHB recently in the USA and Canada, South America, Asia, and Europe (Xu and Chen, 1993, Parry et al., 1995, McMullen et al., 1997). These epidemics in nature could strike suddenly, but its appearance could be inconsistent in years, requiring high humidity and rainfall during flowering in the presence of susceptible host and aggressive strains of the pathogen.

The saprophytic part of the life cycle of *G. zeae* occurs on maize stubbles and provides a reservoir of inoculum for the next season (Sutton, 1982). Wheat field management includes reduced tillage that prevent soil erosion and shortened wheat-corn rotations that provide maximum growing of the two crops. These practices may have

enhanced adaptation of pathogen population by selecting for aggressive strains and provided favorable environment which resulted in disease outbreaks (Teich and Hamilton, 1985). Prospects to control FHB by chemicals were poor because no fungicides have been found so far to be effective in controlling the disease (Milus, 1994). Resistance breeding is the most economical, environmentally friendly and effective way to control the disease (Schroeder and Christensen, 1963). Fusarium head blight resistance is quantitatively inherited with a considerable genetic variation among breeding materials (Mesterhazy, 1995, Miedaner, 1997).

G. zeae produces trichothecene type A toxins (HT-2 toxin, T-2 toxin) and type B toxins (deoxynivalenol, 3-acetylnivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenone-X, calonectrin) and zearalenone (Marasas et al., 1984, D'Mello and MacDonald, 1997). Type B toxins could be subdivided into two major chemotypes: (1) nivalenol chemotype which produces nivalenol (NIV) and fusarenone-X and (2) deoxynivalenol chemotype which produces deoxynivalenol (DON, vomitoxin) and acetyldeoxynivalenol (ADON). As a member of the sesquiterpenoid family of natural products, trichothecene toxins such as DON are potent eukaryotic protein inhibitor (Sharma and Kim, 1991, Snijders, 1994) causing decreased food consumption and weight gain in non-ruminant animals followed by diarrhea, vomiting, reproductive and haematological problems (Ueno, 1987). Human ingestion of contaminated grains is associated with alimentary toxic aleukia, nausea, depression of the immune system, skin necrosis and hemorrhage of lungs and gastrointestinal tract (Marasas et al., 1984, Beardall and Miller, 1994). Trichothecenes are also phytotoxins causing chlorosis, necrosis, and wilting (McClean, 1996). For this reason, these compounds might contribute to the pathogenicity and/or aggressiveness of the pathogen. DON producers of *G. zeae and F. culmorum* are considered more aggressive on wheat and rye than NIV producers (Gang et al., 1998, Cumagun et al., 2004).

G. zeae is homothallic, i.e., a single mycelium is capable of reproducing sexually, but can be outcrossed under laboratory conditions to allow meiotic analysis (Bowden and Leslie, 1999). Outcrossing has not yet been directly observed in the field, but has been speculated on data from laboratory studies using phenotypic markers such as vegetative compatibility groups (VCGs) (Bowden and Leslie, 1992) and molecular markers (Schilling et al., 1997, Miedaner et al. 2001). Based on the sequence analysis of six genes, O'Donnell et al. (2000) classified the fungus into seven biogeographically structured lineages which may eventually be regarded as species. These lineages have come from different regions of the world: Lineages 1 to 5 coming from the Southern Hemisphere (two in South/Central

America, three in Africa) and the recently derived lineage 6 and 7 from the Northern Hemisphere, lineage 6 being restricted to Asia (Japan). An eighth lineage from Brazil has been recently discovered (Ward et al., 2002). At least four of these lineages can cause typical FHB disease in wheat. Lineage 7, being the most dominant, is the only lineage that has been so far reported in Europe and North America.

As used in our studies, the term pathogenicity means the ability to cause disease while aggressiveness refers to the quantity of disease induced by a pathogenic isolate on a susceptible host in a system in which the isolates do not interact differentially with host cultivars (Vanderplank, 1968). Aggressiveness of *G. zeae* is non-host specific, i.e., races have not been found in populations of this fungus (Mesterhazy, 1995, Van Eeuwijk et al., 1995). Quantitatively varying levels of aggressiveness have been demonstrated in *G. zeae* in field and international collections (Miedaner and Schilling, 1996, Miedaner et al., 2001). High genotypic diversity in a collection of 24 isolates from 23 locations in Kansas, each belonging to different VCGs (Bowden and Leslie, 1992) and even from single wheat heads (Bowden and Leslie, 1997) was reported. Miedaner et al. (2001), using random amplified polymorphic DNA (RAPD) markers, found high degree of variation among isolates within species from different countries. Sources of genetic variability are mutation, somatic recombination by heterokaryosis and sexual recombination that are likely due to large population sizes and short asexual generation times of *G. zeae*. Additionally, genetic variation in fungal populations can be affected by genetic drift, gene flow, and selection (McDonald and McDermott, 1993). It has ever been a challenge to investigate to which extent variations in pathogen aggressiveness and mycotoxin production are due to genetic or non-genetic effects.

Little is understood about genetics of pathogenicity and aggressiveness in *G. zeae*. So far, only trichothecenes have been conclusively identified as an aggressiveness factor by gene technology. This was done by disrupting *TRI5,* the gene that encodes trichodiene synthase, the first enzyme in the trichothecene gene cluster to generate a trichothecene-deficient isolate (Proctor et al., 1995). The inability to produce trichothecenes did not affect pathogenicity in wheat or maize but aggressiveness by producing significantly less disease on both hosts (Proctor et al, 1995, Desjardins et al., 1996, Harris et al., 1999, Bai et al., 2002). In addition to trichothecenes, the role of cell-wall degrading enzymes, hormones and metabolites including zearalenone and fusarins on aggressiveness cannot be ruled out (Miedaner, 1997, Wanyoike et al., 2003). Recently, a mitogen-activated protein kinase homologue Gpmk1 has been identified as pathogenicity factor (Jenczmionka et al., 2003).

Advances in genomics to identify and characterize genes involved in these traits have also been initiated. Kruger et al. (2002) established an EST (expressed sequence tag)-data base comprising of many sequences, unique to wheat scab interaction. To further enrich the database and improve gene characterization, Trail et al. (2003) have recently generated 7996 ESTs from three cDNA libraries and identified 2110 putative genes of the fungus.

Use of segregating populations of *G. zeae* offers an alternative approach to study pathogenicity and aggressiveness of *G. zeae* by analysis of the inheritance of these traits including mycotoxin production. Jurgenson et al. (2002) constructed the first amplified fragment length polymorphism (AFLP)-based genetic map of *G. zeae* consisting of 1048 markers that mapped to 468 loci on nine linkage groups. The application of this map has become evident in the **first part** of our research with the aim to map pathogenicity and aggressiveness in an interlineage cross of a low NIV- producing isolate R-5470 from Japan and high DON-producing isolate Z-3639 from Kansas, USA, estimate the number of QTLs responsible for these traits and determine if either pathogenicity or aggressiveness in the greenhouse is associated with the type or amount of mycotoxin produced under laboratory conditions. The **second part** aims to analyse polymorphisms by AFLP and RAPD markers within lineage 7 of two medium DON producing parents (FG24 from Szeged, Hungary and FG3211 from Sersheim, Germany) and their progeny and compare genetic similarity of the parents of the different lineages. The **third part** aims to analyse the inheritance of aggressiveness traits (head blight rating and relative plot yield) and DON production of the progeny of the same intralineage cross and estimate the relative size of environmental, progeny and progeny-environment interaction variance of these traits across three field environments. The **fourth part** takes into account the covariation between fungal colonization (measured as *Fusarium* exoantigen content) and DON production of *G. zeae* on wheat plants and provide adequate evaluation of fungal aggressiveness. The **fifth part** provides insights on the stability of aggressiveness in a segregating population of *G. zeae* in both greenhouse and field environment which could eventually contribute to minimal costs in testing aggressiveness for plant breeders, pathologists and biotechnologists.

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Genetic Mapping of Pathogenicity and Aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) Toward Wheat

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Accepted for publication 27 January 2004.

ABSTRACT

Cumagun, C. J. R., Bowden, R. L., Jurgenson, J. E., Leslie, J. F., and Miedaner, T. 2003. Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. Phytopathology 93: 000-000.

Gibberella zeae is the major fungal pathogen of Fusarium head blight of wheat and produces several mycotoxins harmful to humans and domesticated animals. We identified loci associated with pathogenicity and aggressiveness on an Amplified Fragment Length Polymorphism (AFLP)-based genetic map of *G. zeae* in a cross between a lineage 6 nivalenol producer from Japan and a lineage 7 deoxynivalenol producer from Kansas. Ninety-nine progeny and the parents were tested in the greenhouse in two years. Progeny segregated qualitatively 61:38 for pathogenicity:nonpathogenicity. The trait maps to linkage group IV adjacent to loci that affect colony pigmentation, perithecium production, and trichothecene toxin amount. Among the 61 pathogenic progeny, the amount of disease induced (aggressiveness) varied quantitatively. Two reproducible quantitative trait loci (QTL) for aggressiveness were detected on linkage group I by simple interval analysis. A QTL linked to the *TRI5* locus (trichodiene synthase in the trichothecene pathway gene cluster) explained 51% of the variation observed and a second QTL some 50 cM away, 29% of the phenotypic variation. *TRI5* is tightly linked to the locus controlling trichothecene toxin type. The two QTLs, however, were likely part of the same QTL by composite interval analysis. Progeny that produced deoxynivalenol were, on average, about twice as aggressive as were those producing nivalenol. No transgressive segregation for aggressiveness was detected. The rather simple inheritance of both traits in this interlineage cross suggests that relatively few loci for pathogenicity or aggressiveness differ between lineage 6 and 7.

Additional keywords: AFLP, cereal, deoxynivalenol, nivalenol, scab, trichothecenes, *Triticum aestivum*

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) causes scab or head blight of wheat and other small grains. As one of the most important plant diseases in the United States and in many other parts of the world (30), it incurs serious economic losses not only in terms of yield (53), but also by contamination of the grain with trichothecene mycotoxins. These compounds are potent inhibitors of eukaryotic protein synthesis, and can cause serious mycotoxicoses in both humans and domesticated animals (29,42). Trichothecenes also are toxic to plants (17), and these compounds are thought to play a role in pathogenicity, i.e., ability to cause disease, aggressiveness, or both, i.e., quantity of disease induced by a pathogenic isolate on a susceptible host (11,17,41).

Genotypic differences among isolates of *G. zeae* from collections and field populations have been extensively described (5,13,31,33,54,55). O'Donnell et al. (38) proposed that the species is divided into a series of seven phylogenetic lineages based on DNA sequences of six genes. Interestingly, sequences of trichothecene pathway genes do not generate the same phylogenies (51). The lineages have different geographic distributions, differ in production of trichothecene mycotoxins, and may differ in their ability to cause disease on particular crops.

Deoxynivalenol (DON) and its acetylated derivatives 3-acetyldeoxynivalenol (3-ADON), and nivalenol (NIV) are the most important trichothecenes found in cereals (29). DON, also known as vomitoxin, is the most common trichothecene in Europe and North America (40). NIV-producing strains of *G. zeae* have been reported in Europe (4), Africa (45), Asia (44), and South America (14) but not in North America (1). The occurrence of high NIV-producing strains that produce little or no DON, and vice versa, is now well established (32), and a single gene (*TRI13*) responsible for the differential ability to produce DON or NIV has been identified (25). NIV is considered to be more toxic to animals than is DON (46).

The role of trichothecenes in plant disease is not clear. A positive correlation between aggressiveness and DON production by *G. zeae* and *F. culmorum* has been reported (15,19), but other results have showed no correlation or have been inconsistent (2,26,50). A trichothecene-deficient *G. zeae* mutant induced by disruption of *TRI5*, the gene encoding the first enzyme in the trichothecene pathway, was still pathogenic to wheat, rye (11,41), and maize (17), but was less aggressive than its wild-type progenitor. Recently, Bai et al. (3) reported that DON-non-producing strains could cause initial infections on wheat spikes, but could not spread beyond the initial infection, suggesting that DON is an aggressiveness, rather than a pathogenicity factor (17, 41). There are several reports that DON-producing strains are more aggressive towards plants than are those that produce NIV (27,32,34); however, there is at least one contrary report (7).

We took an alternative approach to studies of pathogenicity and aggressiveness of *G. zeae* by utilizing the progeny of a cross used to generate a previously constructed genetic map of *G. zeae* (21). The mapping cross was made between a lineage 6 nivalenol producer and a lineage 7 deoxynivalenol producer. The existing genetic map makes it possible to perform quantitative trait locus (QTL) analysis on the progeny. In addition, because the progeny segregate for both the amount and type of trichothecene toxin produced, we could test the hypothesis that these traits are related to either the pathogenicity or the aggressiveness of the strain.

Our objectives in this study were: (i) to estimate the number of QTLs responsible for pathogenicity and aggressiveness and to locate the QTLs on the existing map of *G. zeae*, and (ii) to determine if either pathogenicity or aggressiveness is associated with the type or amount of mycotoxin produced under laboratory conditions. A preliminary report of this study has been published (10).

MATERIALS AND METHODS

Mapping population. The mapping population analyzed is the same as that used by Jurgenson et al. (21) to create a genetic map for *G. zeae*. A DON-producing strain, Z-3639 [member of lineage 7 (38)], isolated from wheat in Kansas (5), and a NIV-producing strain, R-5470 (member of lineage 6 (38), isolated from barley in Japan, and provided by Paul E. Nelson (Department of Plant Pathology, Pennsylvania State University, University Park, PA) served as the parents. The map is based on the segregation of 1048 polymorphic Amplified Fragment Length Polymorphism (AFLP) markers to 468 loci in 99 haploid progeny. The total map length is approximately 1300 centiMorgans (cM) with nine linkage groups and an average distance between loci of 2.8 cM.

Inoculum production. *G. zeae* was cultured on synthetic nutrient-poor agar (SNA; Nirenberg, 1981) in 90 mm diameter Petri dishes that were incubated at 24°C in the dark for the first 24 h and afterwards exposed to two black light tubes (Philips TLO, 40 W/80, Royal Philips Electronics, Amsterdam) for 25/22°C (day/night) for 1-2 weeks until conidia formed. Not all strains produced sufficient conidia under these conditions. These strains grew as flat pionnotal colonies that conidiated when cultured on potato dextrose agar (PDA, Merck, Darmstadt, Germany). Conidia were washed from the plates with sterile tap water and the concentration adjusted to 5×10^4 spores/ml following a count in a haemacytometer.

Greenhouse tests and evaluation. Seeds of a susceptible German spring wheat genotype, Munk, were sown in plastic trays and cultivated in a greenhouse. After approximately 10 days, seedlings were transplanted in groups of six into plastic pots (13 × 13 cm^2) containing compost soil and cultivated in a cool greenhouse with no temperature regulation. Plants were fertilized with nitrogen during the growing stage. Four plant heads/pot for a total of five pots/strain were inoculated at mid-anthesis. Approximately 10 µl of a spore suspension was injected into the left and right floret of a central spikelet on both sides of each head (4 × 10 µl injections per head) with a hypodermic needle (0.5 mm gauge) in a controlled plant growth chamber (21°C day/19°C night). Inoculated plants were covered with plastic sheets and incubated for 48 h in the dark (90-100% relative humidity), then uncovered and incubated for an additional 48 h (60-80% relative humidity) under artificial light. Due to limited space in the plant growth chamber, the progeny were chosen randomly and tested in 3-5 batches with both parental strains included in each batch. After the first four days of incubation, plants were transferred to a heated greenhouse with a mean temperature of 18-20°C and a day length (artificial light) of 16 h. The number of infected spikelets was counted 11, 14, 18 and 23 days after inoculation and calculated relative to the total number of spikelets per head. To minimize error, results from the four evaluation dates for each experiment were averaged (= raw values) and the means adjusted relative to the respective batch mean consisting of 10-24 pathogenic progeny per batch. This experiment was conducted over two years (2001, 2002). Toxin data were the same as used for producing the genetic map (21).

Statistical analyses. Data analyses were based on pot means, i.e., the mean of four heads. When the entire set of progeny was analyzed, the data were not normally distributed (Fig. 1). Therefore analysis of variance (ANOVA) was calculated only for the pathogenic progeny. In the pathogenic progeny subpopulation, error variances were homogeneous across years according to Bartlett's test (43). The two years were treated as a series of random environments according to Cochran and Cox (9). Estimates of variance components were calculated as described by Snedecor and Cochran (43). Broad-sense heritabilities (H^2) were estimated on an entry-mean basis (12) as the proportion of genotypic to phenotypic variance. All analyses were performed with the computer package PLABSTAT (H. Friedrich Utz, University of Hohenheim, Stuttgart). The effects of genotypes, replicates and years were assumed to be random variables. Data were analyzed for both raw and adjusted disease severity.

Initial detection of QTLs for pathogenicity or aggressiveness was done with Map Manager QTX11 software (28) and MAPMAKER for MacIntosh (24). Markers were subjected to

simple interval analysis (16) using QGENE (35) to identify significant associations between AFLP markers and aggressiveness with a significance level at Logarithm of Odds (LOD) 3.0 (23). Relationships between QTLs were investigated by composite interval mapping using PLABQTL (47). Two separate analyses were run: all progeny, and pathogenic progeny only. QTL analyses were performed with adjusted disease severity values only.

RESULTS

Assessment of pathogenicity and aggressiveness. Disease severity increased with time (Table 1), with Z-3639 always the most aggressive (26% of the spikelets was infected 11 days post-inoculation to 53% of the spikelets infected 23 days post-inoculation). The mean values for the pathogenic progeny followed a similar pattern (18-36% infected spikelets over this 12-day period). R-5470 and the 38 non-pathogenic progeny were always the least aggressive, with the number of infected spikelets at 11 days post-inoculation < 0.3%. This value increased to only ~1.6% by 23 days post-inoculation.

The frequency distribution of disease severity appeared bimodal (Fig. 1). The Kansas parental strain, Z-3639, was the most aggressive entry (average adjusted disease severity 215% across years) and the Japanese strain, R-5470, was essentially non-pathogenic (average adjusted disease severity 4.3% across years). Thirty-eight progeny were not pathogenic (adjusted disease severity < 25%) with the members of this subpopulation reacting similarly to the Japanese parental strain. Sixty-one of the progeny were pathogenic in both years (adjusted mean disease severity ≥ 25%). The 61 pathogenic progeny varied in their disease severity, which was interpreted as differences in aggressiveness. Segregation was similar in both years and no transgressive segregants were observed.

Significant (P= 0.01) differences in aggressiveness (Fig. 1) were confirmed in an ANOVA of the results from the pathogenic progeny (Table 2). Progeny × year interaction also was significant ($P < 0.01$). Estimates of the broad-sense heritability (H^2) of aggressiveness across both years for raw and adjusted values were 0.82 and 0.81 (Table 2). The variances observed for both the raw and the adjusted data were similar in proportion.

Chromosomal localization and gene action and interaction. When the set of all progeny was analyzed by single marker regression, several markers on linkage group (LG) IV, including three phenotypic markers, *PER1* (perithecial production), *PIG1* (red pigment production), and *TOX1* (trichothecene toxin amount), had highly significant LODs for marker class means for disease severity (Table 3). Simple interval analysis revealed a large peak near

PIG1 and another similar peak around locus *4P* in both years. Composite interval analysis showed that the peak at *4P* decreased (2002) or disappeared (2001) when background effects at *PIG1* were controlled. This suggested that the two peaks are related. When pathogenicity was treated as a qualitative trait segregating 61:38 (new locus was designated *PATH1*), it mapped adjacent to *PIG1*, *PER1*, and *TOX1* (Fig. 2). The mapping program also could place *PATH1* near locus *4P* with slightly lower LOD. This again suggested that the two regions of LG IV are related. No other significant QTLs on other linkage groups were detected in the analysis of the full set of progeny.

When only the pathogenic progeny were analyzed by single marker regression, none of the markers on LG IV had a significant LOD. However, a large region on LG I, including markers *TRI5* and *EAAMTG0665K*, were associated with aggressiveness (Table 3). Differences in marker class means associated with these QTLs were smaller than the differences associated with the markers on LG IV, but still statistically significant ($P < 0.01$). Simple interval analysis revealed a QTL peak near *TRI5* and a smaller peak near *EAAMTG0655K* in both years (Fig. 3). Composite interval analysis showed that the peak at *EAAMTG0655K* disappeared in both years when background effects at *TRI5* were controlled. No reproducible QTLs were detected on any of the other seven linkage groups.

The two QTLs on LG I explain 51 and 29%, respectively, of the observed variation for aggressiveness in this cross (Table 3). The alleles for nonpathogenicity and for lower aggressiveness all originated from the R-5470 parent, although some recombinants can be noted in each case (Table 4). The LG I-1 and LG I-2 QTLs for aggressiveness are not additive, since the mean disease severity (as adjusted percent infected spikelets), 62-68%, is the same for those with neither or either one of the two Z-3639 alleles, while the mean disease severity for progeny with both the LG I-1 and LG I-2 Z-3639 alleles is 131% (Table 4).

Relationship of toxin production to aggressiveness. Amongst the 99 progeny from this cross, 54 were previously classified as moderate- to high-level producers and 45 were classified as very low producers of trichothecene mycotoxins *in vitro* (21). Toxin amount was strongly related to disease severity, with all of the nonpathogenic progeny classified as low-level producers (Fig. 4A). The adjusted mean disease severity for high-level producers was 98% and the mean for low-level producers was 18%, which was significantly different ($P < 0.0001$). Fifty-four high-level producing progeny were classified for ability to produce nivalenol or deoxynivalenol (Fig. 4B). Twenty-six strains that produced nivalenol and twenty-eight that produced deoxynivalenol had adjusted mean disease severities of 131% and 67%, respectively ($P < 0.0001$).

DISCUSSION

This study is the first to assign specific chromosomal regions in *G. zeae* to differences in disease severity using QTL analysis. Most QTL analyses have been made in plant or animal systems, although there are a few studies with fungi, e.g. Hawthorne et al. (18) and Welz and Leonard (52). We exploited an existing relatively dense genetic map (21) based on 99 progeny from a wide cross of a lineage 6 nivalenol producer from Japan and a lineage 7 deoxynivalenol producer from Kansas.

On the basis of a two-year greenhouse experiment, pathogenicity consistently segregated in a qualitative manner, 61:38. The pathogenicity locus in *G. zeae* was designated *PATH1* and mapped to LG IV (Fig. 2). Even under very favorable conditions for disease, nonpathogenic strains could only rarely spread beyond the inoculated spikelets. Although inheritance of pathogenicity as a single Mendelian gene has been reported in other fungi (e.g. *Cochliobolus carbonum* (36), nonpathogenicity is an unusual character for field isolates of *G. zeae* (31, 32). The segregation ratio for *PATH1* was significantly different from 1:1 ($P = 0.02$). Segregation ratios on LG IV are known to be distorted in this cross due to a putative chromosomal rearrangement and selection for a *nit* marker that was required to make the cross (21). The rearrangement could explain the observation of two related peaks on LG IV and the ambiguity of the linkage relationships for *PATH1*. Since the second peak was reduced or removed by composite interval analysis, it is likely that it is part of the same QTL as the first peak. If *PATH1* was at or near the breakpoints of the putative inversion on LG IV, there could appear to be two loci due to artifacts of the mapping process. Interestingly, when nonpathogenic progeny were removed from the analysis, no QTLs on LG IV could be detected. Therefore, the pathogenicity locus on LG IV was important for pathogenicity, but not for aggressiveness.

The parental strains differ in several additional phenotypic traits including pigmentation (*PIG1*) and level of toxin produced (*TOX1*) that map on linkage group IV (21). Pigmentation and pathogenicity are correlated in several plant pathogens including *Colletotrichum lagenarium* (39), *Nectria haematococca* (18), and *Magnaporthe grisea* (8), and *PIG1* was the marker most closely linked to *PATH1*. The level of toxin produced has been reported as a pathogenicity factor in the interaction between *G. zeae* and wheat and maize (3,11,17,41). The *TOX1* locus is associated with the amount of toxin produced *in vitro*, maps near *PATH1* on LG IV, and might play a role in determining pathogenicity. The large difference in disease severity between high toxin producers and low producers favors that hypothesis. The absence of progeny that produce high levels of deoxynivalenol or nivalenol, but that are

nonpathogenic also supports the hypothesis. However, the existence of progeny (Fig. 4A) that produce low levels of toxin *in vitro*, but that are highly pathogenic argues against that hypothesis. It is possible that some progeny genetically capable of being high producers failed to produce high toxin levels *in vitro*. In that case, *TOX1* and *PATH1* could, in fact, be the same locus. It should be noted, however, that the type of toxin, but not the amount of toxin correlated between field and *in vitro* data (15).

Hou et al. (20) recently reported that a mitogen-activated protein kinase gene (*MGV1*) in *G. zeae* is involved in processes related to sexual reproduction and pathogenicity. Both the Japanese parental strain, R-5470, and the *MGV1* mutant (20) produce low levels of toxin, have reduced female fertility, and are non-pathogenic. Thus, R-5470 may carry a pleiotropic mutation similar to that in *MGV1*.

If the pathogenic progeny are evaluated as an independent subset, then the variation remaining for mean disease severity is still significant, and the effect of the locus on LG IV is masked. Under these conditions, two additional linked QTLs were identified on LG I that account for 51 and 29% of the variation associated with this trait in this cross (Table 3, Fig. 3). Since the smaller peak was removed in both years by composite interval analysis, it is likely part of the same QTL. We consider the differences affected by this QTL to be differences in aggressiveness *sensu* Vanderplank (48). Although quantitative differences in disease severity have been reported for field isolates of *G. zeae* (31-33), this study is the first to demonstrate quantitative differences in aggressiveness in a segregating population and to evaluate the genetic basis for these differences. QTL analysis clearly distinguished these loci for aggressiveness on LG I from those controlling pathogenicity on LG IV.

Interestingly, the *TRI5* gene, which encodes the enzyme trichodiene synthase (6) in the trichothecene biosynthetic pathway gene cluster in *G. zeae*, was closely linked to QTL LG I-1 (Table 3). *TRI5* presumably serves as a marker for the trichothecene cluster in which *TRI13*, the gene that determines whether nivalenol or deoxynivalenol will be produced (25), also resides. As far as we can tell, the genes in the trichothecene cluster other than *TRI13* are all functional in both strains. The *TRI13* allele from Z-3639 is presumably non-functional, which leads to the production of deoxynivalenol instead of nivalenol. On average, the deoxynivalenol-producing progeny from our cross were about twice as aggressive on wheat as the nivalenol-producing progeny (Fig. 4B). This result is consistent with correlations observed in field collections (27,32,34). Thus, our data are consistent with the hypothesis that the QTL for aggressiveness on LG I results from allelic differences at one or more of the loci in the trichothecene gene cluster, probably *TRI13*.

Previous studies (32,33) suggested that aggressiveness is a continuous character and therefore probably the result of numerous quantitative genes. In contrast, this study suggests that only two reproducible loci affecting disease severity were segregating in this wide cross between lineage 6 and 7. We also found no evidence of transgressive segregation, which would support the hypothesis that many quantitative genes control aggressiveness. Still, some aggressiveness QTLs could have been missed in this study. First, it is possible that the parents were not polymorphic for some important QTLs. Second, the statistical power of the analysis is reduced by the relatively small number of pathogenic progeny and the segregation distortion on LGs II, IV, V, and VI. Third, some QTL may have been masked by environmental variables or might be more readily detected in field trials instead of greenhouse experiments. Additional mapping populations with more progeny might enable us to detect other QTLs that have lesser effects and perhaps map elsewhere in the genome. To assess accurately the effect of aggressiveness factors other than toxin type and remove toxin production from consideration as a pathogenicity factor, a cross between strains that produce similar levels of either deoxynivalenol or nivalenol but differ in the level of disease severity should be analyzed.

Our results may have implications for the evolution of more aggressive *G. zeae* populations. We expected to see some transgressive segregation in the progeny. However, none of the progeny were more aggressive than the lineage 7, deoxynivalenol parent. Therefore, the risk of nivalenol-producing immigrants resulting in highly aggressive new strains into regions dominated by DON producers may be low. If highly resistant wheat genotypes are grown on a large scale, aggressiveness might increase in the pathogen population as a whole. Specific wheat cultivar × fungal strain interactions are unknown in this organism (49), but the lack of observed interactions may be due, at least in part, to ignorance of the genetics of the pathogen. Now that a QTL for aggressiveness has been confirmed on LG I, it is possible to look for cultivar × strain interactions, e.g. by testing the aggressiveness of nivalenol and deoxynivalenol strains on highly resistant wheat varieties. Thus, QTL mapping of these traits in the *G. zeae* could identify genes involved in specific interactions between the host and the pathogen and provide basic information needed for the management of both host and pathogen populations within the wheat agro-ecosystem.

ACKNOWLEDGEMENTS

We thank Bianca Schneider and the staff of the rye working group at the University of Hohenheim for technical assistance. C. J. R. Cumagun was supported by a Ph.D. Fellowship from the German Academic Exchange Service (DAAD), Bonn. This work was supported in part by the US Wheat and Barley Scab Initiative and the Kansas Agricultural Research Station, Manhattan. Contribution no. 04-069-J from the Kansas Agricultural Experiment Station, Manhattan.

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TABLE 1. Mean disease severity across two years (percentage of infected spikelets, raw values) in the greenhouse 11, 14, 18, and 23 days after inoculation of *Gibberella zeae* strains Z-3639 and R-5470, and the mean of 61 pathogenic and 38 non-pathogenic progeny of a cross between these two strains

Entry	Days after inoculation[y]			
	11	14	18	23
Z-3639	26 a	39 a	47 a	53 a
Pathogenic progeny	18 a	24 b	29 b	36 a
Least significant difference ($P = 0.05$)	11	15	17	23
R-5470	0.3	0.4	0.7	1.6
Non-pathogenic progeny[z]	0.1	0.2	0.4	1.0

[y] Numbers followed by different letters in the same column are significantly different at $P = 0.05$.

[z] Analysis of variance was not calculated because the frequency distribution deviates from normality.

TABLE 2. Variance component estimates and entry-mean heritabilities of disease severity of 61 pathogenic progeny of *Gibberella zeae* with raw values and following normalization to the respective batch means across two years

Parameter	DF	Raw values	Adjusted
Sources of variation			
Year (Y)	1	4.96	-[x]
Progeny (P)	60	127 **[y]	1500 **
P x Y	60	50 **	600 **
Pooled Error	487	25	590
Heritability (H^2)		0.82	0.81
90% C.I. for H^2[z]		0.70-0.89	0.69-0.89

[x] Negative estimate.

[y] ** Significant at $P = 0.01$ (F-test).

[z] Confidence intervals (C.I.) on H^2 were calculated by the method of Knapp and Bridges (22).

TABLE 3. Marker and marker position, phenotypic and genetic effects, maximum LOD scores, and proportions of phenotypic variance explained by markers (R^2) for percentage infected spikelets in progeny of *Gibberella zeae* cross Z-3639 × R-5470 in two subsamples tested in the greenhouse for two experimental years

Subsample	Linkage group region	Nearest marker	Marker Position[x] (cM)	Phenotypic difference of marker classes[y] Z-3639	R-5470	LOD	R^2 [%][z]	Additive genetic effect
All progeny	LG IV-1	*TOX1*	24	99	18	12	43	-76
		PER1	33	100	10	16	51	-84
		PIG1	40	100	2.3	20	60	-91
		4B	43	96	9.2	15	50	-84
	LG IV-2	*4P*	100	98	7.3	18	56	-89
Pathogenic progeny only	LG I-1	*TRI5*	111	130	66	9.6	51	-63
	LG I-2	*EAAMTG0655K*	156	118	65	4.6	29	-50

[x] Based on existing genetic map of *G. zeae* (21).

[y] All marker class differences were significant at $P < 0.001$. Adjusted mean disease severity expressed as percentage of infected spikelets following normalization to the respective batch means from two different years.

[z] R^2 = Percent phenotypic variance explained by each locus using single marker regression analysis.

TABLE 4. Marker class means for adjusted mean percentage infected spikelets at loci *TRI5* and *EAAMTG0655K* on linkage group I for 61 pathogenic progeny from *Gibberella zeae* cross Z-3639 × R-5470 tested in the greenhouse in two different years

	TRI5[y]		
EAAMTG0655K	Z-3639	R-5470	Mean
Z-3639	131 (31)[z]	68 (10)	100 (41)
R-5470	66 (1)	62 (19)	64 (20)
Mean	99 (32)	65 (29)	

[y] Percentage infected spikelets.

[z] Numbers in parentheses indicate number of progeny represented in mean.

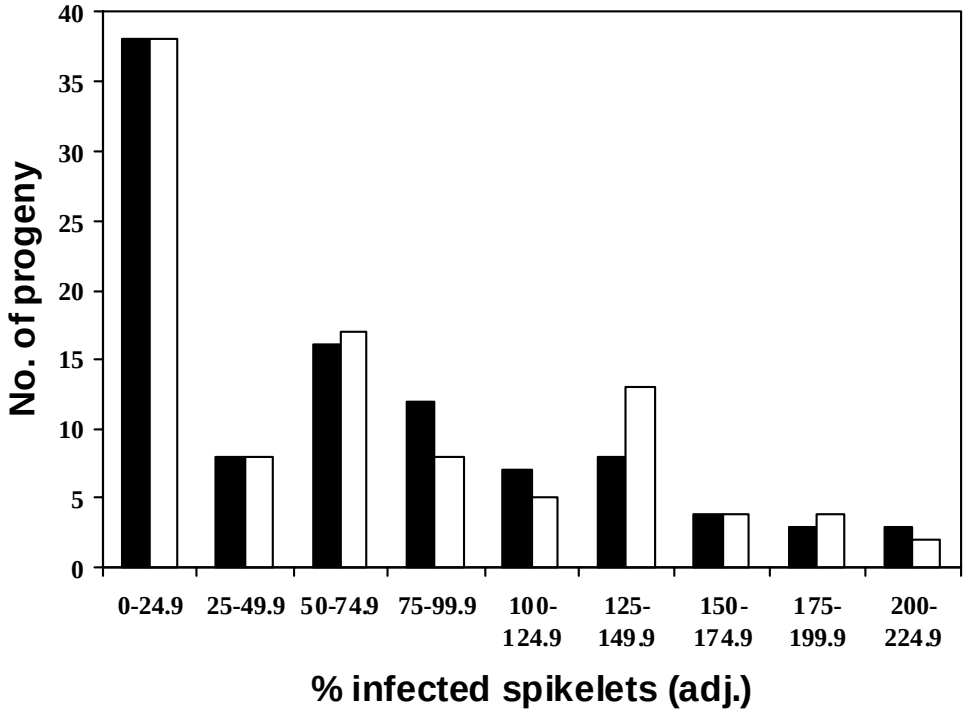

Fig. 1. Frequency distribution of disease severity, percentage of infected spikelets adjusted to the batch mean, for 99 progeny from the cross of *Gibberella zeae* strains Z-3639 (parental mean 215%) and R-5470 (4.3%) in the greenhouse in 2001 (■) and 2002 (□). The least aggressive group contains all 38 of the nonpathogenic progeny.

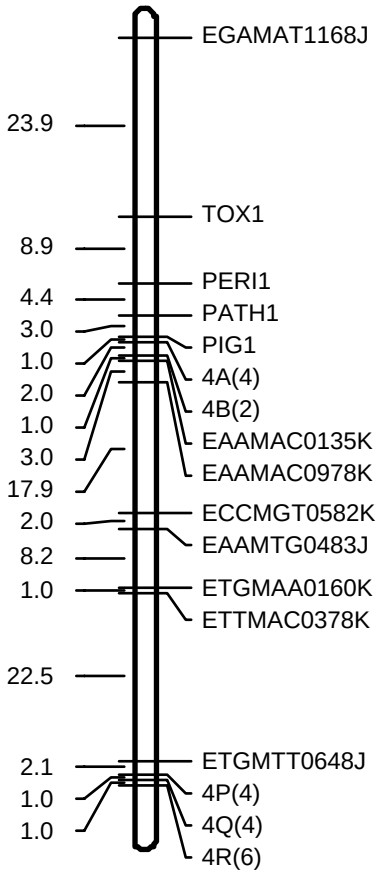

Fig. 2. Location of qualitative gene (*PATH1*) controlling pathogenicity on linkage group IV based on segregation in 99 progeny in the greenhouse in 2001 and 2002.

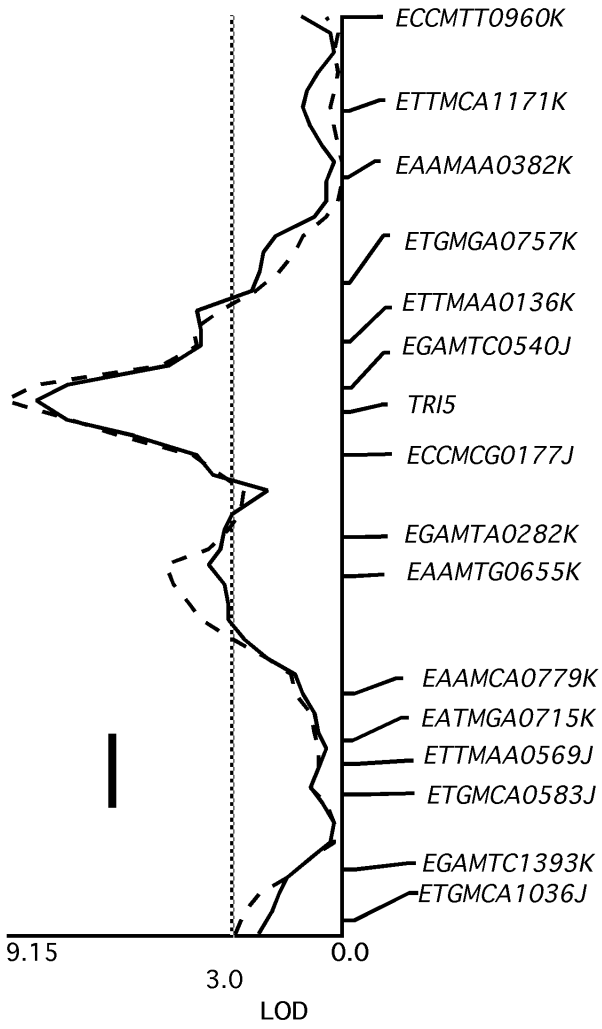

Fig. 3. Location of QTLs for aggressiveness on linkage group I (LG I) of *Gibberella zeae* based on segregation in 61 pathogenic progeny tested in the greenhouse in 2001 (dotted line) and 2002 (solid line). Bar indicates 20 cM. The LOD significance likelihood of 3.0 is marked.

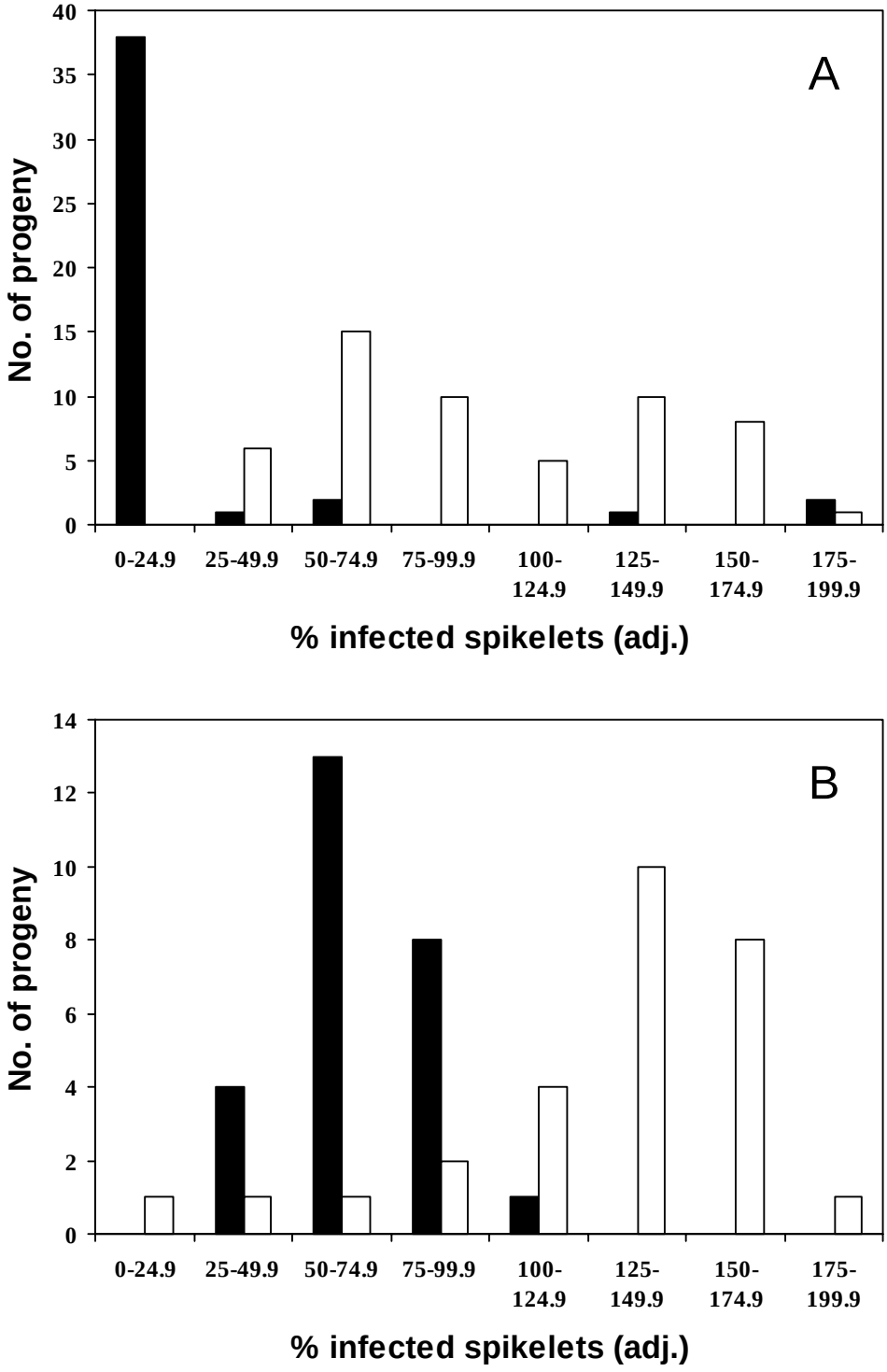

Fig. 4. Frequency distributions of infected spikelets (%) adjusted to their respective batch means in wheat cultivar Munk inoculated with progeny from the cross of *Gibberella zeae* strains Z-3639 (high DON producer) and R-5470 (low NIV producer) in the greenhouse across two experimental years. A. High (□) or low (■) toxin production scored for all progeny (21). B. Deoxynivalenol (□) or nivalenol (■) scored for high toxin producers only (21).

Genetic variation and segregation of DNA polymorphisms in *Gibberella zeae* detected with AFLP and RAPD markers

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ABSTRACT

A parent cross between two deoxynivalenol-producing *Gibberella zeae* FG24 (Szeged, Hungary) x FG3211 (Sersheim, Germany) belonging to lineage 7 was analysed for segregation of polymorphic markers among 153 progeny using Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD). Fifty six RAPD primers and 31 AFLP primer combinations were screened for polymorphism between the parents. High proportion of segregation distortion among progeny was observed using selected primers. Genetic distance of the two parents was compared to isolates lineage 7, Z-3639 (Kansas) and lineage 6, R-5470 (Japan). Rate of polymorphism between Z-3639 and R-5470 was about three to four times greater than between FG24 and FG3211. Isolate Z-3639 was closely associated to FG24 and FG3211 whereas R-5470 was genetically separated based on cluster analysis, thus confirming their lineage grouping. Genetic distances among the four parents using AFLP and RAPD markers were correlated, but association between molecular markers and the aggressiveness of *G. zeae* population could not be established.

INTRODUCTION

Fusarium head blight, caused by *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is a destructive disease of wheat, causing reduction in yield and impairing quality of grains by contamination of toxicogenic mycotoxins such as deoxynivalenol (DON), its derivative 3-acetyldeoxynivalenol (3-ADON), and nivalenol (NIV), which are harmful to humans and animals (Marasas et al., 1984; McMullen et al., 1997). The role of these mycotoxins have been implicated as aggressiveness factors in plant pathogenesis (Proctor et al., 1995; Bai et al., 2002).

Genetic variation of aggressiveness among isolates of *G. zeae* collected within a single field or from different geographical areas is commonly reported (Miedaner and Schilling, 1996; Miedaner et al., 2001; Muthomi et al., 2002), but the mechanisms of such variation is not well understood. The application of PCR-based technologies such as Amplified Fragment Length Polymorphisms (AFLP) and Random Amplified Polymorphic DNA (RAPD) and other DNA based markers have facilitated greatly the genetic analyses of phytopathogenic fungi (Brown et al., 1996). Based on the DNA sequences of six genes, O'Donnell et al. (2000) proposed that *G. zeae* consists of seven phylogeographical lineages coming from different geographical origins which may eventually be considered as species. Recently, a high-density genetic linkage map of *G. zeae* has been published (Jurgenson et al., 2002). The map was constructed from an interlineage cross between Kansas parent Z-3639 and Japanese parent R-5470 generating 99 progeny. Analysis of Quantitative Trait Loci (QTL) associated with pathogenicity and aggressiveness of this population has been done recently (Cumagun et al., 2004). For linkage mapping and QTL analysis, we attempted to use another population from an intralineage cross between two DON-producing parents FG24 (Hungary) and FG3211 (Germany) with 153 progeny. Both isolates were characterized by pink white colony, red pigmentation on potato dextrose agar (PDA), and aerial growth habit. Aggressiveness and DON production of the two parents did not differ greatly, but we expected that these characters will segregate quantitatively in the progeny. Based on classification proposed by O'Donnell et al. (2000), these isolates belong to lineage 7. Map construction, however, has been laborious and unsuccessful due to the monomorphic character of the parents and the high frequency of segregation distortion, i.e., markers deviating from the Mendelian ratio. We therefore resorted simply to assessing the polymorphism of progeny population

by AFLP and RAPD markers and comparing the genetic similarity and distance of the two parents with Z-3639 and R-5470.

MATERIALS AND METHODS

Crossing population

Two pairs of parents, FG24 (Hungary) x FG3211 (Germany) and Z-3639 (Kansas) x R-5470 (Japan), were crossed in the lab of B. Bowden and J. Leslie at Kansas State University, USA (Bowden and Leslie, 1999). FG24, FG 3211, and Z-3639 belong to lineage 7 and R-5470 to lineage 6 (O' Donnell et al., 2000). Only the first parent cross consisting of 153 progeny was analysed for segregation.

Culture media, DNA extraction and quantification

One hundred fifty seven isolates of *G. zeae* (including the four parents) were routinely cultivated in SNA (synthetic nutrient-poor mineral agar) according to Nirenberg (1981). Mycelia of the parent isolates and their progeny were produced in 100 ml flasks containing 20 ml of liquid SNA (without agar), and supplemented with 0.1% yeast extract and 10-fold increase of sugars. A 100 ml flask was inoculated with 1-3 mycelial plugs of a vigorously growing culture of each isolate and incubated at room temperature with natural light for 4 to 6 days on a shaker at 100 rpm. After incubation, pure mycelia were filtered off from the liquid culture on filter paper disks using a Buechner type funnel and a filter flask connected to a water jet pump. Mycelia were washed once with sterile-distilled water on the filter paper and scraped off after excess liquid had been removed. Mycelia were immediately frozen at –20 ^{o}C for storage and then freeze-dried for 48 h prior to DNA extraction. Dried mycelia were crushed into a fine powder in a mixer-mill MM2 (Retsch, Haan, Germany) at 80 rpm for 30 sec.

Total genomic DNA was isolated from 50 mg mycelium by a microextraction protocol according to Möller *et al*. (1992) including treatment with RNase A. The reaction tube consists of powdered mycelium with 500 µl TES (Tris-EDTA-SDS) (100 m*M* Tris, pH 8.0, 10 m*M* EDTA, 2% SDS) and 50-100 µg Proteinase K from an appropriate stock solution. The reaction was incubated for 30 min (minimum) up to 1 h at 55°-60°C with occasional gentle mixing. Salt concentration was adjusted to 1.4 *M* with 5 *M* NaCl (= 140 µl), added with 1/10 vol (= 65 µl) 10% CTAB and incubated for 10 min at 65°C. One vol SEVAG (= 700 µl) (chloroform: isoamylalcohol, 24:1) was added, mixed gently, incubated for 30 min at 0°C and centrifuged for 10 min at 4°C, rpm_{max}.

Supernatant was transferred to a 1.5 ml tube, added with 225 µl 5 *M* NH_4Ac, placed on ice for approx. 30 min and centrifuged, 4°C, rpm_{max} for 15 min. Supernatant was then transferred to a fresh tube; 5 µl RNase (10 mg/ml) was added and incubated for 37°C. A 0.55 vol isopropanol (= 510 µl) was added to precipitate DNA and centrifuged immediately for 5 min, rpm_{max}. Pellets, upon removal of supernatant, were washed twice with cold 70% ethanol, air-dried, and dissolved in 50 µl TE (Tris-EDTA).

DNA was quantified by diluting stock DNA with 1:10 and 1:50 with water and afterwards run in an electrophoresis chamber for 3 h at 50 V. Bands were stained with ethidium bromide and photographed under UV. Intensity of bands were quantified using standard digested Lambda DNA.

AFLP analysis

AFLP analysis was based on Vos et al. (1995) with some modifications using non-radioactive staining (Zhong and Steffenson, 2001). AFLP core reagent kit (Life Technologies, Inc., Grand Island, NY) was used to digest and ligate DNA as template for PCR. AFLP primers (Life Technologies Inc., Bethesda, MD) with one selective base were used in preamplification. The sequences of the preamplification primers were: 5'-CTC GTA GAC TGC GTA CCA ATT C-3' (E + C) and 5'-GAC GAT GAG TCC TGA GTA A-3' (M + A). Amplification was performed in a 46 µl reaction volume using a Thermo Cycler (MJ Research Inc. Watertown MA), programmed for 20 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and 94°C for 30 s. The PCR reaction contained 25mM dNTP (deoxynucleotide triphosphates) stock, 10x *Taq* polymerase buffer, 5 units *Taq* DNA polymerase, 50 ng each of *Eco*RI and *Mse*I preamplification primer, and a 5-fold dilution of template DNA. Preamplification products were run in 1% agarose gel at 85 V for 90 min to check the success of amplification. Primers with two selective bases were used for selective amplification (Table 1). The amplification reactions were performed in a 20 µl reaction volume, containing 10x PCR buffer, 5 units *Taq* DNA polymerase, 27.8 ng *Eco*RI, 6.7 ng *Mse*I + 0.89 µM dNTPs, and a 5-fold dilution of the preamplified DNA template. The PCR reaction consisted of 94°C for 30 s, 65°C for 30 s with a step-down of annealing temperature by 0.7 C/cycle, followed by 12 cycles at 72°C for 1 min, 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, 94°C for 30 s and finally 22 cycles at 56°C for 30 s and 72°C for 5 min.

After the amplification reactions, PCR products were added with an equal volume of formamide dye to each sample, denatured by heating for 3 min 90°C and quickly

chilled on ice immediately prior to gel loading. A 4.4 µl sample was loaded onto an 6% polyacrylamide gels (Sequa Gel, Biozym, Oldendorf) including Low Mass Ladder™ (Life Technologies Inc., Bethesda, MD) as a standard size marker. Electrophoresis was performed initially for 5 min at 1000 V (pre-heating) and 1200 V for 3 h. Gels were visualized by silver staining (DNA Silver Staining System, Promega, Madison, WI). AFLP bands from 100 to 2000 bp in size were scored using TotalLab v1.10 software package (Nonlinear Dynamics. Ltd, Newcastle upon Tyne).

RAPD analysis

RAPD analysis was according to Schilling et al. (1994) using RAPD decamer primer kit # UBC 100/1 (University of British Columbia, Vancouver) and OPT (Operon Technologies, Alameda, CA) with their sequences as shown in Table 2. PCR amplifications were conducted in a 50-µl reactions containing 1 x *Taq* polymerase buffer (50 m*M* KCl, 10 m*M* Tris, pH 7.5, 1.5 m*M* $MgCl_2$), 25 m*M* $MgCl_2$, 5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Germany), 1 m*M* of dNTP, and 5 m*M* of each decamer primer.

Three cycle profiles were as follows: 94°C for 3 min, 35°C for 1 min, and 72°C for 2 min (cycle 1); 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min (cycles 2-44); and a final cycle of 5 min at 72°C. Samples were prepared for electrophoresis by adding 5 µl gel loading buffer. Reaction mix (11 µl) was loaded and DNA fragments were resolved by electrophoresis (2 V cm^{-1}) in 1.5% agarose and 1 x TAE (Tris-acetate-EDTA) for 5 h aside with a 200 bp O'RangeRuler DNA ladder (MBI Fermentas, Germany). Gels were stained in ethidium bromide and photographed under UV light with a Polaroid camera.

***TRI5* analysis**

Ten ng of genomic DNA from each parent and progeny were digested with 4 units of *Mse*I (New England BioLabs, Beverly, MA) for 2 h. Digested DNA were amplified with two primers: 5'-GGCATGGTTGTATACAGC–3' and 5'CAGAGTGATCTCATGG CAGG–3' and run on 1% agarose gel at 75 V for 2 hr. Gels were stained and visualized as described previously.

Data analyses

AFLP and RAPD bands were scored manually and analysed as binary data with 1 (band present) and 2 (band absent) at a particular location in each lane. Using the program

Tools for Population Genetic Analyses (TFPGA) version 1.3 (Miller, 1997), genetic similarity between isolates were calculated (Nei, 1972). A dendrogram was constructed using the unweighted pair-group method (UPGMA) following Wright's (1978) modification of Roger's (1972) distance.

RESULTS AND DISCUSSION

The number of AFLP and RAPD polymorphic markers in the *G. zeae* parent isolates Z-3639 and R-5470 was about four times as much as in FG24 and FG3211 (Table 1 and 2), which is consistent with the rate of polymorphism (Table 3). One to 12 polymorphic markers per AFLP primer combination within 100 bp to 2000 bp and one to four per RAPD primer were detected between FG24 and FG3211. Genetic similarity between these two parents was quite high despite their geographical separation as compared to Z-3639 and R-5470 (Fig. 1, Table 4). Dendrograms generated from both AFLP and RAPD markers showed that the two European parents closely resembled the Kansas parent confirming their lineage grouping while the Japanese parent was obviously far distant from the three parent isolates, thus belonging to a separate lineage (Fig. 2A and 2B). Molecular analysis was also associated with the cultural characters of the four parents. About 50% difference of the observed AFLP bands between the Kansas and Japanese parent is consistent with that of Jurgenson et al. (2002), although we used silver staining method.

A total of 71-122 bands per lane were generated using AFLP compared to 1-11 using RAPD. AFLP examines the whole genome and is highly reproducible (Vos et al., 1995). These features make AFLP a more reliable technique than RAPD. Reproducibility is due to the specificity of primer annealing and is homologous to the adapter sequence and the restriction site sequence. RAPD has been critized for lack of reproducibility (Williams et al., 1990). We found, however, a good association between the two markers although the two methods of analysis could reveal genetic variation in different regions of the genome. Despite the advantages of AFLP, use of additional primer combinations could not detect enough polymorphism between FG24 and FG3211. This is consistent to the finding that some isolates of *G. zeae* coming from Southern and Eastern Europe are closely associated based on their principle coordinate analysis (Schilling, 1996). A total of 31 AFLP primer combinations were screened for polymorphisms between the parents

FG24 and FG3211 (Table 1 and Table 3), from which only six primer combinations that generated the highest polymorphisms were used for the progeny population. For RAPD analysis, only four (UBC 23, 29, 30, and 43) out of 56 primers, which generated two to four polymorphic markers, were selected for the progeny population. Both marker systems showed a very low polymorphism between the two parents in this cross and high segregation distortion among the progeny which made it difficult to construct the map. Bowden et al., (2002) encountered the same problem using a narrow cross within lineage 7. The Kansas parent Z-3639 was paired with PH-1 from Michigan. To solve the problem of segregation distortion associated with the *nit* marker technique, one parent that had a deletion in the *MAT2* (mating type 2) gene was used which made it heterothallic. This technique avoided segregation distortion associated with *nit* markers. Segregation distortion was also reported in the Z-3639 x R-5470 cross (Jurgenson et al., 2002). This problem was confirmed in the FG24 x FG3211 cross where out of 45 AFLP polymorphic markers detected, only 28 loci (62%) segregated in a 1:1 Mendelian ratio. It is even worse in the case of RAPD, where one out of nine loci has a 1:1 segregation distortion. (data not shown). Segregation distortion was also observed in *Phytophthora infestans* and *Leptosphaeria maculans* (Van der Lee et al., 1997; Pongam et al., 1998). This is a common phenomenon in linkage analysis when linkage between markers and genes favors or acts against the survival of the individual progeny. Haploid organisms are more affected because the selection that causes the distortion can act virtually during the whole haploid life cycle (Pedersen et al., 2002). Segregation distortion could also be due to differential viability of ascospores isolated from different progeny genotypes. Extending the length of the PCR primers may reduce segregation distortion for AFLP markers (Nikaido et al., 1999). Frequent linkages among AFLP markers were also observed in our study.

Electrophoretic analysis of the *TRI5* fragment digested with *Mse*I revealed monomorphic bands between the parents and the progeny, suggesting genetic homology between the European isolates for this locus. There was no association between phenotypic data (aggressiveness) from three field environments and molecular markers. Similarly, variation in aggressiveness and mycotoxin production was not related to variation in RAPD patterns (Miedaner et. al., 2001; Muthomi et al., 2002). The degree of correlation between virulence and molecular markers is often low in populations that reproduce sexually (Burdon and Roelfs, 1985), suggesting that DNA analysis provides a

weak predictive information about the variation of aggressiveness and the potential for new pathotypes to evolve.

Overall, the high genetic similarity we found between the two European parents limits contruction of a genetic map. As a fallback plan, we recommend to test polymorphisms of a collection of *G. zeae* isolates within lineage 7. It is possible that we selected the monomorphic FG24 and FG 3211 parents by chance. If lineage 7 isolates are highly monomorphic indeed, it would be worth to continue mapping the FG24 x FG3211 cross despite the huge amount of work required.

ACKNOWLEDGEMENTS

We thank Andrea Merz for doing the RAPD analysis. This research was funded by the German Academic Exchange Service (DAAD).

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Table 1. Primers used for AFLP analysis of *Gibberella zeae* isolates and number of polymorhic bands for two pairs of isolates

Primer combination	Sequence 5'-3'	No of polymorphic bands (100-2000 bp)	
		FG24/FG3211	Z-3639/R-5470
E-AA+M-AA	AGA CTG CGT ACC AAT TCA A GAT GAG TCC TGA GTA AAA	1	17
E-AT+M-CA*	AGA CTG CGT ACC AAT TCA T GAT GAG TCC TGA GTA ACA	5	35
E-GA+M-AA	AGA CTG CGT ACC AAT TCG A GAT GAG TCC TGA GTA AAA	2	8
E-TG+M-AG	AGA CTG CGT ACC AAT TCT G GAT GAG TCC TGA GTA AAG	3	11
E-AA+M-AG	AGA CTG CGT ACC AAT TCA A GAT GAG TCC TGA GTA AAG	2	ND[b]
E-GA+M-TA*	AGA CTG CGT ACC AAT TCG A GAT GAG TCC TGA GTA ATA	3	14
E-TT+M-AA	AGA CTG CGT ACC AAT TCT T GAT GAG TCC TGA GTA AAA	3	ND
E-TT+M-AC	AGA CTG CGT ACC AAT TCT T AGA CTG CGT ACC AAT TCA C	2	ND
E-CG+M-CG	AGA CTG CGT ACC AAT TCC G GAT GAG TCC TGA GTA ACG	3	18
E-GA+M-AA	AGA CTG CGT ACC AAT TCG A GAT GAG TCC TGA GTA AAA	NP[a]	5
E-GA+M-TC	AGA CTG CGT ACC AAT TCG A GAT GAG TCC TGA GTA ATC	3	15
E-TG+M-AG	AGA CTG CGT ACC AAT TCT G GAT GAG TCC TGA GTA AAG	NP	19
E-AA+M-CA	AGA CTG CGT ACC AAT TCA A GAT GAG TCC TGA GTA ACA	NP	16
E-CC+M-AT	AGA CTG CGT ACC AAT TCC C GAT GAG TCC TGA GTA AAT	3	15
E-CC+M-TT	AGA CTG CGT ACC AAT TCC C GAT GAG TCC TGA GTA ATT	2	NP
E-GA+M-AT*	AGA CTG CGT ACC AAT TCG A GAT GAG TCC TGA GTA AAT	7	33
E-TG+M-AA	AGA CTG CGT ACC AAT TCT G GAT GAG TCC TGA GTA AAA	1	17
E-AA+M-AC	AGA CTG CGT ACC AAT TCA A AGA CTG CGT ACC AAT TCA C	2	19
E-AT+M-CG	AGA CTG CGT ACC AAT TCA T GAT GAG TCC TGA GTA ACG	3	14
E-CC+M-GT	AGA CTG CGT ACC AAT TCC C GAT GAG TCC TGA GTA AGT	0	4
E-GC+M-GT	AGA CTG CGT ACC AAT TCG C GAT GAG TCC TGA GTA AGT	NP	NP
E-TG+M-GA	AGA CTG CGT ACC AAT TCT G GAT GAG TCC TGA GTA AGA	NP	NP
E-AA+M-CC	AGA CTG CGT ACC AAT TCA A GAT GAG TCC TGA GTA ACC	3	NP

Table 1. (continued)

Primer combination	Sequence 5'-3'	No of polymorphic bands (100-2000 bp)	
		FG24/FG3211	Z-3639/R-5470
E-AA+M-TG	AGA CTG CGT ACC AAT TCA A GAT GAG TCC TGA GTA ATG	1	10
E-AT+M-GA	AGA CTG CGT ACC AAT TCA T GAT GAG TCC TGA GTA AGA	NP	NP
E-GC+M-AG*	AGA CTG CGT ACC AAT TCG C GAT GAG TCC TGA GTA AAG	12	10
E-GC+M-TC	AGA CTG CGT ACC AAT TCG C GAT GAG TCC TGA GTA ATC	1	4
E-TT+M-CC	AGA CTG CGT ACC AAT TCT T GAT GAG TCC TGA GTA ACC	NP	NP
E-AA+M-AT	AGA CTG CGT ACC AAT TCA A GAT GAG TCC TGA GTA AAT	3	19
E-CC+M-CG*	AGA CTG CGT ACC AAT TCC C GAT GAG TCC TGA GTA ACG	9	20
E- TG+M-TT*	AGA CTG CGT ACC AAT TCT G GAT GAG TCC TGA GTA ATT	10	NP
Total		84	323

[a]NP, no amplification products.

[b]ND, not determined.

*Selected for further analysis (N = 6).

Table 2. RAPD primers used for analysis of *Gibberella zeae* and number of bands polymorphic for two pairs of isolates

Primer[a]	Sequence 5'-3'	No of polymorphic bands	
		FG24/FG3211	Z-3639/R-5470
UBC1	CCTGGGCTTC	1	2
UBC2	CCTGGGCTTG	1	6
UBC3	CCTGGGCTTTA	NP[b]	2
UBC4	CCTGGGCTGG	0	2
UBC5	CCTGGGTTCC	NP	NP
UBC6	CCTGGGCCTA	0	2
UBC7	CCTGGGGGTT	NP	NP
UBC8	CCTGGCGGTA	0	1
UBC9	CCTGCGCTTA	NP	NP
UBC10	GGGGGGATTA	NP	NP
UBC12	CCTGGGTCCA	1	6
UBC13	CCTGGGTGGA	2	5
UBC14	CCTGGGTTTC	NP	NP
UBC15	CCTGGGTTTG	0	NP
UBC17	CCTGGGCCTC	0	4
UBC18	GGGCCGTTTA	0	NP
UBC20	TCCGGGTTTG	NP	NP
UBC21	ACCGGGTTTC	0	NP
UBC22	CCCTTGGGGG	NP	NP
UBC23*	CCCGCCTTCC	2	8
UBC24	ACAGGGGTGA	0	NP
UBC25	ACAGGGCTCA	1	7
UBC26	TTTGGGCCCA	NP	NP
UBC27	TTTGGGGGGA	NP	NP
UBC28	CCGGCCTTAA	NP	3
UBC29*	CCGGCCTTAC	3	NP
UBC30*	CCGGCCTTAG	4	3
UBC32	GGGGCCTTAA	1	3
UBC33	CCGGCTGGAA	0	1
UBC34	CCGGCCCCAA	0	4
UBC35	CCGGGGTTAA	0	2
UBC36	CCCCCCTTAG	NP	NP

Table 2. (continued)

Primer[a]	Sequence 5'-3'	No of polymorphic bands	
		FG24/FG3211	Z-3639/R-5470
UBC37	CCGGGGTTTT	1	NP
UBC38	CCGGGGAAAA	0	NP
UBC39	TTAACCGGGC	NP	0
UBC40	TTACCTGGGC	0	0
UBC41	TTAACCGGGC	0	NP
UBC42	TTAACCCGGC	1	1
UBC43*	AAAACCGGGC	2	2
UBC44	TTACCCCGGC	0	0
UBC45	TTAACCCCGG	0	NP
UBC46	TTAAGGGGGC	NP	NP
UBC47	TTCCCCAAGC	NP	NP
UBC48	TTAACGGGGA	NP	NP
UBC49	TTCCCCGAGC	0	1
UBC50	TTCCCCGCGC	0	NP
UBC59	TTCCGGGTGC	0	2
UBC66	GAGGGCGTGA	0	2
UBC72	GAGCACGGGA	0	4
UBC77	GAGCACCAGG	0	0
UBC78	GAGCACTAGC	0	3
UBC85	GTGCTCGTGC	0	0
UBC90	GGGGGTTAGG	2	NP
UBC98	ATCCTGCCAG	0	2
OPT16	GGTGAACGCT	0	0
OPT19	GTCCGTATGG	NP	NP
Total		22	78

[a]Primer code (University of British Columbia, Vancouver, Canada; Operon Technologies, Alameda, CA).

[b]NP, no amplification products.

*Selected for further analysis (N = 4)

Table 3. Rate of polymorphism detected by AFLP and RAPD analysis in FG24/FG3211 and Z-3639 / R-5470 crossing parents for selected AFLP primer combinations

Category	AFLP		RAPD	
	FG24/ FG3211	Z-3639/ R-5470	FG24/ FG3211	Z-3639/ R-5470
Number of primers tested	6	5	36	31
Total number of fragments detected	446	415	191	206
Average number of fragments detected/primer combination	74	83	5.3	6.6
Number of polymorphic fragments	46	122	22	78
Rate of polymorphism (%)	10.3	29.4	11.6	37.9
Average number of polymorphic fragments/primer combination	7.6	24.4	0.6	2.5

Table 4. Genetic similarity of the parents using AFLP and RAPD markers according to Nei (1972)

Parents	Genetic similarity	
	AFLP [a]	RAPD [b]
FG24/FG3211	0.93	0.89
Z-3639/R-5470	0.65	0.47

[a]Based on 6 AFLP primer combinations.

[b]Based on 36 RAPD primers.

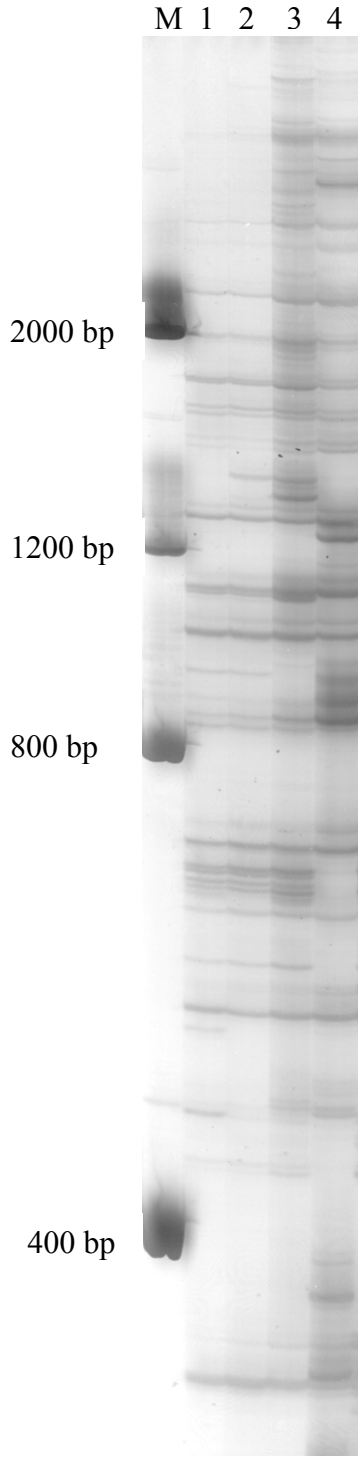

Fig. 1. AFLP gel showing low polymorphism between FG24 (lane 1) and FG3211 (lane 2) in contrast with Z-3639 (lane 3) and R-5470 (lane 4) amplified with primer combination E-GA+M-AT. Molecular marker (M) is a 2000 bp ladder.

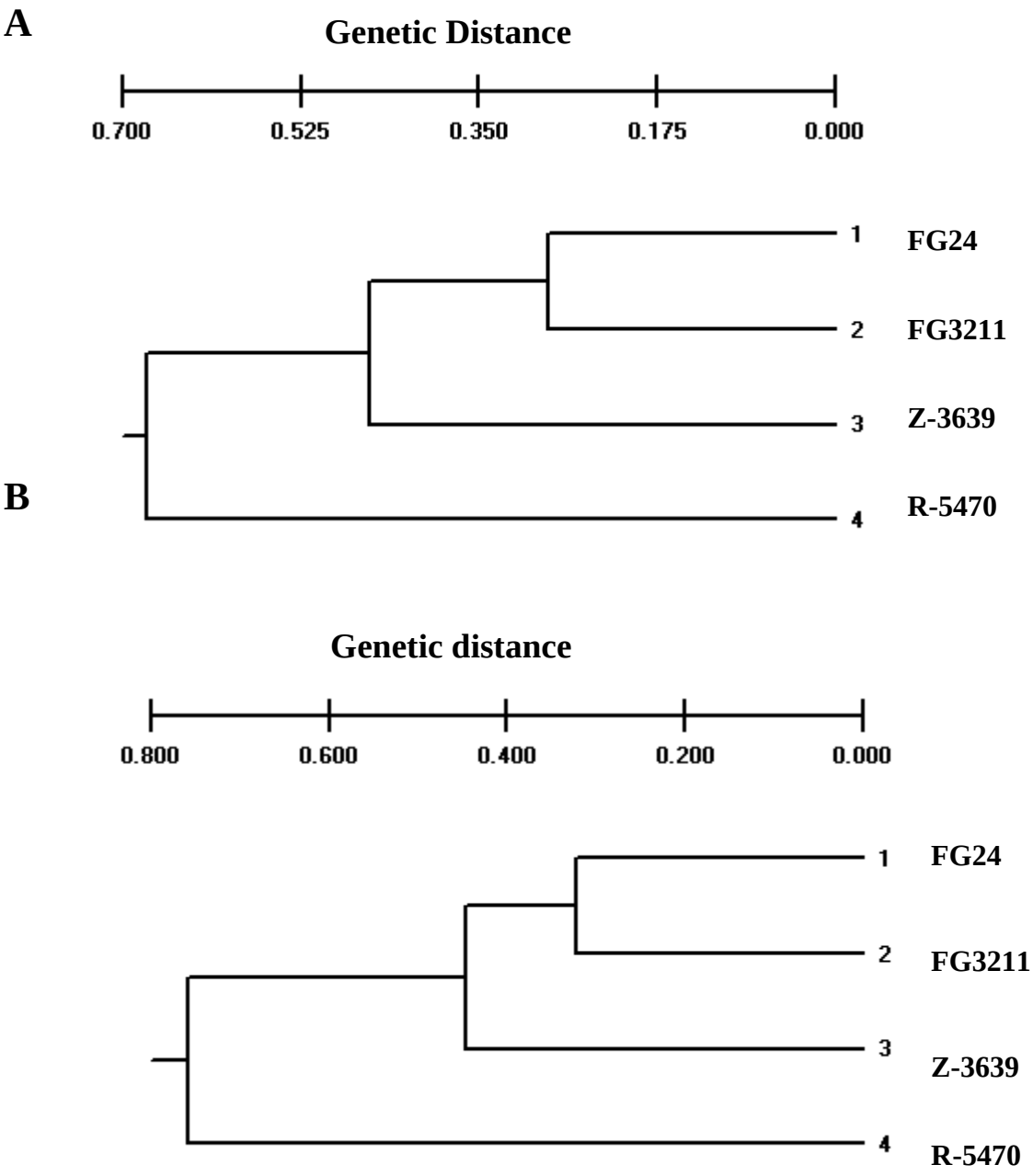

Fig. 2. Dendrograms of four parents of *Gibberella zeae* based on AFLP (A) and RAPD markers (B). Genetic distances were revealed by UPGMA cluster analysis using Roger's distance (1972) and Wright's (1978) modification. Genetic distances for AFLP and RAPD were calculated from the combined data of six primer combinations and 26 primers, respectively.

European Journal of Plant Pathology **00**:1-11, 2004
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Segregation for aggressiveness and deoxynivalenol production of a population of *Gibberella zeae* causing head blight of wheat

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Accepted 14 February 2004

Key words: inheritance, mycotoxin, quantitative variation, scab

Abstract

Gibberella zeae is a devastating pathogen of wheat and other small-grain cereals, causing yield losses and reducing grain quality by producing the trichothecene deoxynivalenol (DON) which is harmful to animals and humans. We analyzed 153 progeny from a cross between two European DON-producing isolates of *G. zeae* for aggressiveness and DON production in three environments (location-year combinations) in Germany. Aggressiveness, measured as head blight rating and relative plot yield, and DON production showed continuous distribution for each environment and across environments. There was significant (P = 0.01) genotypic variation for all three traits. Transgressive segregants occurred for all three traits. Both repeatability estimates within an environment and heritability estimates on an entry-mean basis for head blight rating and DON production were medium to high (0.5-0.7). Progeny-environment interaction accounted for about 29% of the total variance for the two aggressiveness traits and 19% for DON production. The large genetic variation derived from a cross between two rather similar European parents indicates a potential for increasing fungal aggressiveness in the *G. zeae* population.

Introduction

Gibberella zeae (anamorph: *Fusarium graminearum* Schwabe) is an important plant pathogen worldwide. The fungus infects several major crops, including maize, wheat, barley, rye, and triticale. In wheat, *G. zeae* causes seedling blight, crown rot, root rot, and head blight. Head blight or scab of wheat causes epidemics in many wheat areas worldwide (Dubin et al., 1997; McMullen et al. 1997). The disease reduces grain quality as well as yield, leading to poor germination of infected grains, reduction in baking quality, and mycotoxin contamination. Deoxynivalenol (DON), and its derivatives 3-acetyl-DON and 15-acetyl-DON, nivalenol (NIV), as well as zearalenone (ZEA), the major mycotoxins produced by *G. zeae* (Marasas et al., 1984), pose animal and human health risks. In North America and Europe, DON is the most frequently encountered mycotoxin (Müller and Schwadorf, 1993; Placinta et al., 1999; Bottalico and Perrone, 2002).

Aggressiveness is the quantity of disease induced by a pathogenic isolate on a susceptible host (Van der Plank, 1968). Several studies indicate that large genetic variation in aggressiveness among *G. zeae* isolates exists as sampled from various parts of the world (Bai and Shaner, 1996; Miedaner et al., 2001), within a country or state (Dusabenyagasani et al.,1999; Walker et al., 2001) and even within populations

* This paper is dedicated to Prof. Dr. H.H. Geiger, Hohenheim, on the occasion of his 65th birthday.

from individual fields (Miedaner and Schilling, 1996). There is also quantitative variation in mycotoxin production of *G. zeae* among a collection of isolates from winter rye (Miedaner et al., 2000). However, little is known about the genetic basis of aggressiveness and DON production by *G. zeae*. Knowledge of genetics of aggressiveness and DON production of *G. zeae* is important in developing resistant cultivars and in estimating durability of resistance. Resistance to *G. zeae* head blight in wheat is quantitatively inherited. All known cultivars are infected but the degree of infection greatly varies (Bai and Shaner, 1996).

Several studies on the role of trichothecenes in plant diseases suggest that DON production increases aggressiveness of *G. zeae* (Proctor et al., 1995; Desjardins et al., 1996; Harris et al., 1999). Proctor et al. (1995) showed that a DON-deficient isolate of *G. zeae* generated by gene disruption remained pathogenic on wheat and rye but was less aggressive than the wild type. The finding that trichothecenes enhance the aggressiveness of *G. zeae* suggests that it could be possible to reduce head blight of wheat and mycotoxin problems caused by this fungus by breeding trichothecene-resistant crops. Disease-causing capacity of the trichothecene-deficient mutant of *G. zeae*, however, indicates that there are other factors that contribute to aggressiveness, such as cell-wall degrading enzymes (Schwarz et al., 2002; Wanyoike et al., 2002) and other metabolites. Studies on host resistance and genetic modification of the pathogen are required to understand the role of DON in plant pathogenesis. Resistant cultivars will remain the most practical and effective control against head blight of wheat (Martin and Johnston, 1982).

Sexual recombination of *G. zeae* has been demonstrated under laboratory conditions, and should also occur in the field as indicated by high genotypic diversity reported within individual fields (Bowden and Leslie, 1992; Miedaner and Schilling, 1996). In the study reported here, a crossing population of *G. zeae* with two DON-producing parents was tested in three field environments to analyze the inheritance of aggressiveness and amount of DON produced and the effect of environmental variation and isolate–environment interaction on aggressivenesss traits and DON production. A preliminary report of this work concerning onefield environment has been published (Cumagun et al., 2002).

Materials and Methods

Crossing population and field tests

Two DON-producing strains of *G. zeae*, FG24 from Szeged, Hungary, and FG3211 from Sersheim, Germany, both isolated by T. Miedaner, served as parents for the crossing population. Both strains belong to lineage 7 (O'Donnell et al., 2000). The parents were chosen from a previous experiment (Miedaner et al., 2000) to represent different aggressiveness and DON production levels. Methods for crossing *G. zeae* were described in detail by Bowden and Leslie (1999). *G. zeae* is homothallic and can outcross or self. Therefore, complementary nitrate non-utilizing (*nit*) mutations were employed as suitable markers. Parents were crossed and single ascospore cultures were isolated in the lab of B. Bowden and J. Leslie at Kansas State University, U.S.A. Moderately susceptible German winter wheat cultivar Drifter was inoculated in three field environments in Southwest Germany: Hohenheim (HOH) near Stuttgart (400 m above sea level, 8.5 ^{o}C mean annual temperature, 685 mm mean annual precipitation) in 2001 and 2002 and Oberer Lindenhof (OLI) near Reutlingen (700m above sea level, 6.6 ^{o}C mean annual temperature, 952 mm mean annual precipitation) in 2002.

Inoculum production, inoculation, field design, disease and yield assessment

Conidia from 153 single-ascospore progeny of the cross of *G. zeae* and the two parents were mass produced following the procedure of Miedaner et al. (1996). Wheat grains (~300 g) previously soaked overnight in tap water were placed in 1 l milk bottles. Bottles were sealed with aluminum foil and autoclaved twice on successive days, at 121^{o}C for 20 min at 1 atm. Cooled wheat medium was inoculated with 10-20 ml of the conidial suspension, prepared by flooding one week-old SNA (synthetic-nutrient poor agar culture, Nirenberg, 1981) of *G. zeae* with sterile distilled water (SDW), and shaken to distribute the inoculum. After incubation at 18^{o}C in the dark for

4 weeks, colonized wheat grainswere taken out of the bottles, mixed with SDW, and placed in a thin layer in plastic trays (40x 60 cm^2). Trays were completely covered with plastic sheets and placed about 40 cm below two black light tubes (Philipps TLO, 40W/80, Royal Philipps Electronics, Amsterdam) for 3 days. Plastic sheets were folded back from part of the tray when wheat grains were colonized by the fungus. Colonized wheat grains were sprayed with sterile water while breaking-up clumps of moldy grains by hand and inoculum was air-dried for 2 to 3 days at room temperature, enclosed in plastic bags, and stored in a cold chamber at 5 ^oC until inoculation. Conidia concentration was adjusted to 500,000 conidia ml^{-1} for each isolate with the use of a haemacytometer. Plants were inoculated with this concentration at a rate of 120 ml m^{-2} at anthesis. Inoculum suspensions were added with one to two drops of liquid cleansing agent to make sprays more efficient. Inoculation was done in three batches because of the large number of progeny. Batch 1 consisted of progeny numbers 1-51; batch 2, progeny numbers 52-102; and batch 3, progeny numbers 103-153. The progeny of the three batches were sprayed onto host plants in three successive days relying on natural moisture.

A split-plot design was used with the batches as main plots and the progeny as subplots in three replicates. Within the main plots, plots inoculated with the two parental isolates consisted of 10 replicates each to increase accuracy in comparing them with their progeny. Uninoculated plots were also included to calculate relative yield components. Subplots were randomized following a complete block design. Three-row microplots were used (1.2 m length and 0.625 m width) for each isolate and plots were arranged in a chess-cross design, i.e., each plot inoculated with an isolate was surrounded by four border plots of similar size planted with triticale to avoid plot-by-plot interference.

Two aggressiveness traits were assessed: head blight rating per plot and plot yield relative to the non-inoculated control. Head blight was rated by visual estimation (0-100%) of the whole plot when differences in head blight severity among treatments could be observed. This rating includes number of heads infected (incidence) and amount of bleached spikelets per head (severity). Due to artificial inoculation, plots were evenly infected according to the aggressiveness of the respective isolates. Timing of the next rating depended upon the rate of disease development from the previous disease rating. In HOH 2001, disease was rated 18, 20, 25, 32, and 44 days after inoculation and in HOH 2002 and OLI 2002, 14, 16, and 21 days after inoculation. Arithmetic means of the head blight ratings of each assessment date were averaged for further analyses. Grain weight was determined by cutting the whole plot by hand, threshing in a small combine, drying the grain to about 13% moisture, sieving to remove fragments of glumes and rachis, and cleaning again. For relative plot yield, yield of each progeny from the inoculated plots was calculated relative to the respective mean of the control plots and expressed in percentage.

Mycotoxin analysis

Wheat grain was ground and mycotoxins were extracted by weighing out 5 g of each sample in 100 ml flasks containing 100 ml double distilled water. Sample suspensions were placed in a rotary shaker (200 rpm) for 5 min and about 1 ml of the suspension was transferred into Eppendorf tubes and centrifuged (14000 rpm) for 5 min. One ml of supernatant served as stock solution for dilution preparations. Dilutions were prepared from the stock solution to optimize ELISA analysis. Mycotoxin content (DON and 3-acetyl-DON) of ground wheat grain samples was analyzed using RIDASCREEN™ FAST DON (R-biopharm GmbH, Darmstadt, Germany), an enzyme immunoassay for the quantitative analysis of deoxynivalenol in cereals, malt, and feed, and a microtiter plate spectrometer (TECAN SLT Lab Instruments, Crailsheim, Germany). Due to high cost of ELISA, only two replicates from each field experiment were analyzed.

Statistical analyses

Plot means were used for analyses of variance. Residuals were independent and followed a normal distribution for head blight rating, but not for relative plot yield and DON production. The latter two trait values were adjusted to normality by square root transformation. The three environments (year-location combinations) were analyzed as a series of random environments according to Cochran and Cox (1957). Estimates of variance components (σ^2) were calculated as described by

Snedecor and Cochran (1989, page 322). An appropriate model for a split-plot design was derived to take into account the partitioning into three batches: $\sigma^2_e + \sigma^2_{r:e} + \sigma^2_b + \sigma^2_{be} + \sigma^2_{br:e} + \sigma^2_{p:b} + \sigma^2_{pe:b} + \sigma^2$, where e = environment, r:e = replicate within environment, b = batch, be = batch-environment interaction, br = batch-replicate interaction, p:b = progeny within batch, pe:b = progeny-environment interaction within batch, and σ^2 = error variance. Coefficient of variation (cv%) of the respective variance components was calculated as square root of the estimate relative to the population mean. This allows direct comparison between trait means of different units. Repeatability estimates were calculated by partitioning the phenotypic variance of spatial replications within one experiment according to the formula $\sigma^2_p / (\sigma^2_p + \sigma^2)$ (Falconer, 1989) where p = progeny. Broad-sense heritabilities (h^2) were estimated on an entry-mean basis (Fehr, 1987) as the ratio of genotypic to phenotypic variance using the formula: $h^2 = \sigma^2_p / (\sigma^2_p + \sigma^2_{pe}/E + \sigma^2_e/RE)$, where *R* = number of replicates and *E* = number of environments. Confidence intervals of heritability were computed according to Knapp and Bridges (1987). All statistical analyses were computed using the statistical package PLABSTAT (Utz, 2000). All effects were assumed to be random variables.

Results

Flowering dates of wheat were different in the three environments. Wheat fields in HOH 2002 flowered one week earlier than in OLI 2002 due to climatic differences. This situation resulted in a one-week difference in inoculation period between the two locations. Mean temperatures, relative air humidity, and total precipitation varied considerably among three field environments and even among batches and days of inoculation (Table 1).

Uninoculated plots had a mean head blight rating of 0.9% and mean DON levels of 1.15 mg kg^{-1} across field environments. Several head blight ratings in the field were taken at successive dates from 14 to 44 days after inoculation depending upon disease progress (Table 2). Disease progress was slightly different among environments. First symptoms appeared in 2002 earlier and were about four times more severe 20-21 days after inoculation than in 2001. All head blight ratings showed genotypic differences at the 1% probability level and were highly intercorrelated ($r \geq 0.7$, data not shown). For the further analyses, therefore, the mean of the three ratings shown was used.

Both parents were rather similar, i.e., low to medium in aggressiveness and DON production (Table 3). Significant differences between the parents were only found in head blight rating and DON production in HOH 2002 and DON production in OLI 2002. Genotypic variation was highly significant in each batch and combined across all batches for mean head blight rating, relative plot yield, and DON production. Mean head blight rating and relative plot yield in HOH across years were relatively stable. Differences in disease levels could be attributed in part to differences in temperature and relative humidity, which influenced infection period. However, mean head blight rating in HOH for both years could not be compared directly in terms of disease severity because of large differences in disease progress in 2001 and 2002 (Table 2). The highest head blight infection occurred in HOH 2002, which could be linked to its highest mean temperature among field environments.

Relative plot yields in HOH 2001 and 2002 were similar and slightly lower in OLI 2002 (Table 3). Lower mean relative plot yield in 2002 than in 2001 is an indication of higher disease severity in HOH 2002 because infection occurred earlier. Mean DON production in HOH 2001 was almost thrice as much as in HOH 2002 although head blight rating and relative plot yield did not differ much. Batch 1 had the lowest disease and DON level for both years in HOH and batch 3 in OLI 2002. Repeatability estimates for each environment were medium to high for head blight rating and DON production and low to medium for relative plot yield.

All progeny of *G. zeae* cross FG24 x FG3211 caused head blight, reduced grain weight, and produced DON. The progeny differed significantly for these traits (Figure 1). There was a wide range of mean head blight rating, relative plot yield, and DON production across environments. The three traits showed a continuous distribution across environments.

Analysis of variance combined across field environments showed a significant genotypic

Table 1. Mean temperature, sum of precipitation, and mean relative air humidity from the first, second, third day after inoculation and from the total incubation period in three field environments

Enivron-ment[a]	Batch	Mean temperature (°C)				Sum of Precipitation (mm)				Mean relative air humidity (%)			
		Day after inoculation			Incubation	Day after inoculation			Incubation	Day after inoculation			Incubation
		First	Second	Third	period	First	Second	Third	period	First	Second	Third	period
HOH 2001	1	15.6	11.5	11.6	15.4	0	6.8	4.4	45.3	68.0	87.0	93.0	72.2
	2	11.5	11.6	11.4	15.8	6.8	4.4	6.4	45.3	87.0	93.0	94.0	71.3
	3	11.6	11.4	11.2	16.2	4.4	6.4	0.7	119	93.0	94.0	75.0	70.6
HOH 2002	1	13.2	14.5	18.4	22.0	5.6	0	0	91.3	73.8	73.7	64.3	70.0
	2	14.5	18.4	21.3	22.4	0	0	0	85.7	73.7	64.3	62.7	68.7
	3	18.4	21.3	22.4	22.8	0	0	0	55.5	64.3	62.7	68.0	67.9
OLI 2002	1	23.8	23.1	20.6	17.8	0.4	0	0	55.2	46.3	49.3	52.3	54.3
	2	23.1	20.6	21.3	17.1	0	0	0	54.8	49.3	52.3	21.7	54.3
	3	20.6	21.3	21.4	16.7	0	0	30	54.8	52.3	21.7	21.3	55.6

Incubation period is the time from inoculation until the first head blight rating.

[a] HOH = Hohenheim near Stuttgart; OLI = Oberer Lindenhof near Reutlingen.

Table 2. Mean head blight rating (%) on successive dates on wheat cultivar Drifter inoculated with 153 progeny of *Gibberella zeae* in three field environments

	Days after inoculation						
Environment [a]	14	16	18	20	21	25	Mean
HOH 2001	- [b]	-	5.1	13.5	-	34.3	33.7 [c]
HOH 2002	28.4	36.4	-	-	46.9	-	37.4
OLI 2002	12.9	24.6	-	-	37.1	-	24.9

[a] HOH = Hohenheim near Stuttgart; OLI = Oberer Lindenhof near Reutlingen.

[b] Not determined.

[c] Mean of five ratings (18, 20, 25, 32, and 44 days after inoculation) with the latter two ratings not shown here.

Table 3. Means, repeatabilities, significance of genotypic variation on wheat cultivar Drifter for head blight rating, relative plot yield, and deoxynivalenol (DON) production inoculated with 153 progeny of *Gibberella zeae* cross FG24 x FG3211 and their parents in three batches of 51 isolates each in three field environments

Environment [a]	Batch/ Parent	Head blight rating (%)		Relative plot yield (%)		DON production (mg kg $^{-1}$)	
		Mean	Repeatability	Mean	Repeatability	Mean	Repeatability
HOH 2001	1	29.0	55.9**	69.0	30.1**	17.7	50.3**
	2	34.6	72.1**	71.1	58.2**	30.0	64.6**
	3	37.6	64.2**	64.3	20.6**	37.2	46.8**
	1-3	33.7	66.9**	68.1	30.1**	28.4	65.5**
	FG24	13.9a [b]	-	80.2a	-	10.3a	-
	FG3211	15.0a	-	90.0a	-	12.3a	-
HOH 2002	1	30.8	45.3**	68.1	45.4**	9.9	53.1**
	2	39.3	71.9**	57.2	58.7**	13.1	37.9**
	3	41.9	66.4**	55.3	39.1**	12.7	58.0**
	1-3	37.4	67.0**	62.6	51.1**	11.9	45.9**
	FG24	10.2a	-	98.7a	-	3.1a	-
	FG3211	37.2b	-	65.8a	-	12.3b	-
OLI 2002	1	27.6	57.1**	55.7	56.1**	12.5	78.9**
	2	27.5	72.6**	53.1	73.7**	15.7	70.0**
	3	19.7	45.2**	55.3	25.4**	17.0	63.9**
	1-3	24.9	59.9**	54.7	54.3**	15.1	71.0**
	FG24	13.4a	-	71.5a	-	8.3a	-
	FG3211	21.6a	-	58.6a	-	9.2b	-

** Significant genotypic variation at probability level $P = 0.01$ (F-test).

[a] HOH = Hohenheim near Stuttgart, OLI = Oberer Lindenhof near Reutlingen.

[b] Numbers followed by the same letter are not significantly different at 5% level for comparison of parents.

variation among progeny within batches for head blight rating, relative plot yield, and DON production (Table 4). The effect of the batch was not significant; however, batches reacted differently according to the environment. An important source of variance was the interaction between progeny and environment within batches, but progeny within batch was also significant. These interactions led to medium heritability estimates for aggressiveness. Error variance was similar for head blight rating and DON concentration and lowest for relative plot yield.

Correlations between head blight rating and relative plot yield (r = -0.9, $P = 0.01$) head blight rating and DON production were high (r = and 0.7, $P = 0.01$). Correlation between relative plot yield and DON production was lower (r = -0.6, $P = 0.01$).

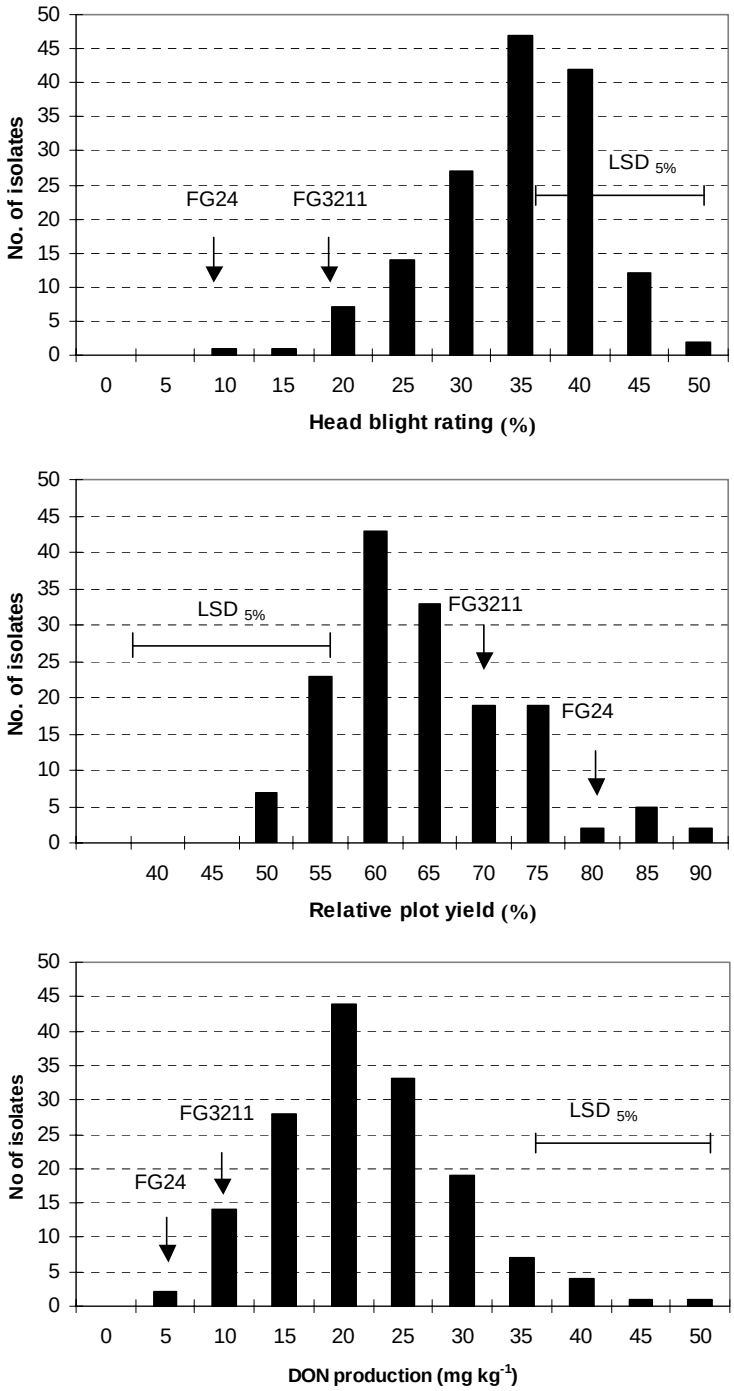

Figure 1. Frequency distribution of head blight rating, relative plot yield, and DON production in the susceptible wheat cultivar Drifter inoculated with 153 progeny of *Gibberella zeae* cross FG24 x FG3211 combined across three field environments (untransformed data); $LSD_{5\%}$ = least significant difference at probability level $P = 0.05$.

Discussion

Significant variance in segregation for aggressiveness and DON production was found among the 153 progeny of a cross between two strains of *G. zeae*, FG24 and FG3211. The parents differed little in these phenotypic characters, although they were collected in different areas, southwestern Germany

Table 4. Coefficients of variation (%) for head blight rating, relative plot yield, and deoxynivalenol (DON) production in the susceptible wheat cultivar Drifter inoculated with 153 progeny of *Gibberella zeae* cross FG24 x FG3211 in three batches of 51 isolates each, calculated across three (aggressive traits) and two (DON production) replicates, respectively, and three field environments.

Parameter	d.f.	Head blight rating	Relative plot yield (transformed)	d.f.	DON production (transformed)
Source of variation:					
Environment (E)	2	16.83	4.90	2	24.84**
Replicate (R): E	6	8.85*	2.92	3	5.58*
Batch (B)	2	- [a]	-	2	10.86
B x E	4	15.24**	1.67	4	8.82**
B x R: E	12	8.52**	2.39**	6	4.08**
Progeny (P):B	150	14.69**	4.25**	150	13.31**
P x E: B	300	22.66**	7.59**	300	16.24**
Pooled Error	897	18.83	9.97	446	17.41
Heritability (h^2)		0.47	0.43		0.67
90% C.I. on h^2 [b]		0.29-0.59	0.25-0.57		0.56-0.75

Data for relative plot yield and DON were normalized by square root transformation
[a] Negative estimate.
[b] Confidence intervals (C.I.) on h^2 were calculated using the method of Knapp and Bridges (1987).
*, ** Significant at probability levels $P = 0.05$, and 0.01, respectively (F-test).

and southern Hungary. Strains FG24 and FG3211 were both DON-chemotype isolates, producing DON in low to medium amounts. Cultural characters were identical, i.e., both were characterized by pink white colony and red pigmentation on potato dextrose agar (PDA) and aerial growth habit (data not shown).There was only a slight difference in aggressiveness. Based on a classification proposed by O'Donnell et al. (2000), both isolates belong to lineage 7 of *F. graminearum.*

The handling of 153 progeny in field inoculations, where the spore concentration of each isolate must be adjusted and each isolate must be applied separately, requires much labor and time. To reduce the workload to a manageable level, we randomly divided the progeny into three batches that were inoculated on three subsequent days. This procedure increased the non-genetic variance due to interactions between batches and environment and batches and replicates (Table 4). In Middle Europe in general, there are daily differences in temperature, rainfall, and humidity that may contribute to this interaction (Table 1). The interaction between progeny and environment within batches was also highly significant. This led to medium-sized heritability for head blight rating, although the repeatabilities of the single batches in the individual environments were medium to high, ranging from 0.5 to 0.7 for the same trait. The latter demonstrates good genotypic differentiation in the individual experiments. Heritability estimate was highest for DON production.

In view of the medium heritabilities obtained, the quantitative variation found for all traits does not necessarily implicate polygenic inheritance. The fact that some progeny were significantly more aggressive, caused lower yields and produced higher DON levels than the most aggressive parent, however, indicates that more than one gene control these traits, and that these genes act additively. These transgressive segregants, comprising almost 80% of the population, still occurred after square-root transformation of the data on relative plot yield and DON production. This implies genetic effects caused by different unlinked alleles for the traits in both parents that recombined in the progeny. This type of inheritance corresponds to the inheritance of the resistance of wheat to *Fusarium* head blight (Snijders 1990; Kolb et al. 2001; Miedaner et al. 2003). The pathosystem reveals a similar quantitative inheritance in both host and pathogen. We have demonstrated this for the first time using a segregating population of *G. zeae*. Quantitative variation of aggressiveness has been reported in other plant pathogenic fungi. For instance, aggressiveness of the smut pathogen *Ustilago hordei* in barley (Emara and Sidhu, 1974) and of *Gaeumannomyces graminis var. triciti* in wheat (Blanch et al., 1981) is quantitatively inherited.

In pathosystems with quantitative variation of aggressiveness and resistance, strong interactions with environment are likely to occur (Dusabenyagasani et al., 1997; Campbell and Lipps, 1998). In particular, *G. zeae* and wheat might react differently to the same environment, thus the effect of genotype x environment interaction of each organism will multiply in infection trials. Indeed, the progeny x environment interaction was one of the most important sources of variance, accounting for 29% of total variance for aggressiveness and 19% for DON production. This finding emphasizes the importance of multi-environmental trials. The highest head-blight rating occurred at HOH 2002, but in HOH 2001 the DON production was two to three times higher.

Correlation between head blight rating and DON production was high. For the latter trait, however, covariation with fungal colonization should be further analyzed because isolates are likely to produce different amounts of mycelium within the same host genotype (Miedaner et al., 2000). The large genotypic variation obtained by crossing two parents from the same lineage implicates a high possibility of adaptation of the pathogen to different environments and hosts by sexual recombination (McDonald and Linde, 2002). Indeed, several studies found a high genotypic variation within individual field populations for aggressiveness, DON production, vegetative compatibility groups, and molecular markers (Bowden and Leslie 1992; Miedaner and Schilling 1996; Miedaner et al. 2001, Walker et al. 2001). According to O' Donnell et al. (2000), all European isolates are lineage 7. Crosses within a lineage are most common and therefore, of highest practical relevance. Crosses between different lineages might occur through global seed trade. Genetic diversity and the potential of the fungus to shift towards greater aggressiveness or toxin production by hybridization within and between lineages should be appreciated (O'Donnell 2000). This general statement could be proven experimentally by our study. As a consequence, even larger genetic variation in *G. zeae* populations would be expected.

The use of aggressive isolates is important in selecting resistant wheat germplasm. Molecular tools permit characterization of genes and quantitative trait loci (QTL) linked to aggressiveness (Hou et al. 2002; Cumagun et al. 2004). Sexual recombination in nature occurs in *G. zeae* regularly by production of perithecia on wheat and on maize stubble in autumn (Sutton 1982; Parry et al., 1995). A maize-wheat crop rotation, as frequently used in Middle Europe and the USA, allows at least one recombination per year and gives the fungus the chance to produce new recombinants. According to population-genetic theory, fungi with mixed recombination, masses of asexually produced conidia, and regularly undergoing sexual recombination (selfing and outcrossing) have the highest risk for adaptation to host resistance (McDonald and Linde, 2002). Because no specific interaction of isolates and host genotypes in *G. zeae* occurs (Van Eeuwijk et al., 1995), directed selection should not play a major role in these populations. On the long term, however, genetic potential for a gradual, unspecific adaptation with increasing aggressiveness levels in populations of *G. zeae* could occur in reaction to host resistance.

Acknowledgements

The excellent technical assistance of Bianca Schneider in the field and the help of the staff of rye working group at the University of Hohenheim is gratefully acknowledged. Special thanks are due to Dr. Robert L. Bowden, Kansas State University, for performing the fungal cross, Sabine Pöschel for rating disease severity and Manfred Buck-Finkele for field assistance at Oberer Lindenhof. C.J.R. Cumagun was supported by a Ph.D. fellowship from the German Academic Exchange Service (DAAD).

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Plant Pathology

Genetic variation and covariation for aggressiveness, deoxynivalenol production and fungal colonization among progeny of *Gibberella zeae* in wheat

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Gibberella zeae causes head blight of cereals and contaminates grains with mycotoxins like deoxynivalenol (DON). To determine the correlations among aggressiveness traits, fungal colonization, and DON production, 50 progeny from a segregating population of *G. zeae* were inoculated onto a susceptible winter wheat cultivar in three field environments. Aggressiveness traits were measured as head blight rating and plot yield relative to noninoculated plots. Fungal colonization, measured as *Fusarium* exoantigen (ExAg) content, and DON production were analysed with two enzyme-linked immunosorbent assay (ELISA) formats. Disease severity was moderate to high based on head blight rating and relative plot yield. *Fusarium* ExAg content and DON production ranged from 0.26 to 1.41 units and from 4.18 to 43.70 mg kg^{-1}, respectively. Significant ($P = 0.01$) genotypic variation was found for all traits. Heritability for *Fusarium* ExAg content was rather low due to high progeny-environment interaction and error. DON/*Fusarium* ExAg ratio did not vary significantly ($P > 0.1$) among progeny. Correlation between DON production and *Fusarium* ExAg content across environments was high ($r = 0.8$, $P = 0.01$), but no covariation existed between aggressiveness traits and DON/*Fusarium* ExAg content ratio.

Key words: aggressiveness, deoxynivalenol, ELISA, exoantigens, *Gibberella zeae*, head blight of wheat

Introduction

Gibberella zeae Schw. (Petch), the perfect stage of *Fusarium graminearum* Schwabe, is a destructive fungal pathogen causing head blight or scab of wheat (Bai & Shaner, 1994) and barley (McMullen *et al*., 1997; Leonard & Bushnell, 2003). The disease is a concern because the fungus causes serious losses in grain yield and infected grains harbour trichothecenes such as deoxynivalenol (DON) which are detrimental to livestock and human health (Marasas *et al*.,1984; Placinta *et al*., 1999).

Although other aggressiveness factors may contribute to the disease, the role of DON has received a considerable attention (Proctor *et al*., 1995; Desjardins *et al.*, 1996; Harris *et al*., 1999). Close association between aggressiveness and DON production has been found earlier for *F. culmorum* (Gang *et al*., 1998) and *F. graminearum* (Miedaner *et al*., 2000, Mesterhazy, 2002). From these studies, however, it is also clear that isolates considerably differ in host colonization. This factor must, therefore, be taken into account. An immunoassay based on exoantigens (ExAgs), a soluble mixture of extracellular fungal products, was first reported by Kaufman & Standard (1987) for measuring fungal biomass within host tissue. Abramson *et al.* (1998) found a linear correlation between ExAgs of *F. graminearum*, detected by an indirect enzyme immunoassay, and DON content (r = 0.76-0.80). A genus specific enzyme-linked immunosorbent assay (ELISA) has also been developed for the detection of *Fusarium* species in cornmeal (Iyer & Cousin, 2003).

Fungal colonization has been found to be a predictive and sensitive indicator of DON (Lamper *et al.*, 2000). Using a competitive PCR assay, Nicholson *et al*. (1998) demonstrated in the field greater colonization on wheat ears by trichothecene-producing wild-type isolate and gene revertants than by non-trichothecene producing isolates of *F. graminearum*. In greenhouse tests, trichothecene-producing isolates caused greater colonization than a non-trichothecene-producing isolate generated by gene disruption (Eudes *et al*., 2001). In some cases, however, fungal biomass *in vivo* was not proportional to the development of disease symptoms and DON production when different field isolates were investigated (Gilbert *et al*., 2001; Asran & Buchenauer, 2003). It is crucial to calculate DON production relative to the amount of fungal biomass in host tissue. Significant genetic variation for these traits have already been reported for

collections of *G. zeae* from different origins (Miedaner *et al.*, 2000; Gilbert *et al.*, 2001; Mesterhazy, 2002) and even within populations derived from individual wheat fields (Miedaner & Schilling, 1996). The tested genotypes, however, were field isolates with no proven genetic relationship. In the present work, we analysed a segregating population of 50 progeny of *G. zeae* to examine the inheritance of and the relationship between fungal biomass (expressed as *Fusarium* ExAg content), disease symptoms, reduction in plot yield, and DON production in wheat grain.

Materials and Methods

Crossing population

Two lineage 7 (O'Donnell *et al*., 2000) and DON-producing strains FG24, isolated from Szeged, Hungary by a co-author, and FG3211 from Sersheim, Germany, served as parents. The choice of the parents was based on differences in aggressiveness and DON production in the field in a previous study across two locations (Miedaner *et al*., 2000). Because *G. zeae* is homothallic, complementary nitrate nonutilizing (*nit*) mutations were employed as suitable markers to distinguish heterozygous from homozygous perithecia. The crossing and single spore isolation were performed in the laboratory of Drs. R. L. Bowden and J.F. Leslie at Kansas State University, USA. Methods for crossing *G. zeae* were described in detail by Bowden & Leslie (1999). Perithecia were produced on carrot agar plates. Plates were inverted and ascospores were collected on the lid. Ascospores were then dilution plated on minimal medium. Progeny were collected as random colonies on minimal medium (containing nitrate as the sole nitrogen source) plates. No more than 20 colonies were saved from each carrot agar plate to reduce the probability of sampling the same meiotic events. We selected only wild type progeny from the cross and discarded *nit* mutant progeny. All progeny were single-spored with a micromanipulator. Fifty single-ascospore progeny, which were pre-selected from 153 progeny, and the two parents were analysed. The pre-selection was carried out in such a way that the distribution of aggressiveness of the original population is represented in the smaller sample (Fig. 1).

Field design

A moderately susceptible German winter wheat cultivar ('Drifter') was planted in three field environments in Southwest Germany: Hohenheim (HOH) near Stuttgart (400 m above sea level, 8.5°C mean annual temperature, 685 mm mean annual precipitation) in 2001 and 2002 and Oberer Lindenhof (OLI) near Reutlingen (700m above sea level, 6.6°C mean annual temperature, 952 mm mean annual precipitation) in 2002. Randomization of plots was according to a complete block design with three replications. Two parental isolates were grown with 10 plots each for higher accuracy of comparison with their progeny. Noninoculated plots for each experiment were grown for assessment of relative yield components. Three-row microplots were used (1.2 m length and 0.625 m width) for each isolate and plots were arranged in a chess-cross design, *i.e.*, each plot with an entry (= isolate) was surrounded by four border plots of similar size and planted with triticale to avoid plot -by-plot interference.

Inoculum production, inoculation and disease assessment

Mass production of isolates was according to the procedure of Miedaner *et al.* (1996). Wheat grains previously soaked overnight in tap water were used as substrate and placed in 1 L milk bottles. Bottles were sealed with aluminum foil and autoclaved twice on successive days, at 121°C for 20 min at 1 atm. Each flask containing cooled wheat medium was inoculated with 10-20 mL of conidial suspension, prepared by flooding a 1-week-old SNA (synthetic-nutrient agar, Nirenberg, 1981) culture with sterile distilled water. Bottles were vigorously shaken to distribute the conidia. After incubation at 16-18°C in the dark for 4 weeks, colonized wheat grains were taken out of the bottles, mixed with sterile distilled water, and placed in a thin layer in plastic trays (40 x 60 cm^2). Trays were completely covered with plastic sheets about 40 cm below two black light tubes (Philips TLO, 40W/80, Royal Philips Electronics, Amsterdam) for 3 days. Sheets were folded from one side of the tray when vigorous sporulation occurred. Colonized wheat grains were mixed to avoid clumping, air-dried for 2 to 3 days at room temperature, enclosed in plastic bags, and stored in a cold chamber at 5°C until inoculation. Concentration was adjusted to 500,000 conidia mL^{-1} for each isolate with the use of a haemacytometer. One to two drops of liquid cleansing agent were added to

inoculum suspensions to reduce surface tension and make sprays more efficient. Plants were inoculated at anthesis using a small hand-held sprayer with a capacity of ~300-500 mL conidial suspension. The sprayer was connected with a hose to a compressor on the tractor to ensure a constant pressure (3 bar) of spray at the rate of 120 ml m^{-2}. All plots reached anthesis simultaneously at a given location.

Two aggressiveness traits were assessed: head blight rating (%) and plot yield relative to noninoculated control (%). Head blight was rated by visual estimation (0-100%) of the whole plot. Rating started when differences in head blight severity among treatments were observed. This rating includes number of heads infected (incidence) and amount of bleached spikelets per head (severity). Timing of the next rating depended upon the rate of disease development from the previous disease rating. In HOH 2001, disease was rated 18, 20, 25, 32, and 44 days after inoculation and in HOH 2002 and OLI 2002, 14, 16, and 21 days after inoculation. Arithmetic means of the head blight ratings of all assessment dates were used for further analyses. Due to artificial inoculation, plots were evenly infected according to the aggressiveness of the respective isolates. Grain weight was determined by harvesting the whole plot by hand, threshing in a small combine, drying to a minimum amount of water content, sieving to remove fragments of glumes and rachis, and cleaning again. For relative plot yield, grain weight of the inoculated plots was calculated relative to the respective mean grain weight of the untreated plots and expressed as a percentage.

Fusarium exoantigen analysis

A serological method developed to quantify fungal biomass in grain samples was used (Rabenstein 2002). For sample preparation, 0.1 g grain flour was grinded in 2 mL extraction buffer (phosphate buffered saline (PBS),without Tween, containing 0.01 *M* ethylenediaminetetraacetic acid disodium salt (EDTA, Sigma code: E 5513) in a mortar and pestle and incubated overnight at 4°C in a refrigerator. Each sample was tested at least two times in each of two replications in an indirect ELISA format already described for the detection of *Rhynchosporium* antigens in barley leaves (Foroughi-Wehr *et al.*, 1995). The methods for antiserum production in rabbits and purification of immunoglobulin (IgG) are essentially as described by Foroughi-Wehr *et al*. (1995). Altogether 8 polyclonal antisera (PAS) were raised in rabbits against surface washings

and/or mycelium homogenates from cultures of *Fusarium culmorum* (Fc*)* and *F. graminearum* (Fg) and the antisera were characterized using ELISA variants (Banks & Cox, 1992, Danks *et al*., 2001) and Western blot analysis (Gan *et al*., 1997). Antiserum PAS Fc 7/2 to soluble ExAgs fractions of *F. culmorum* which revealed in ELISA a strong reaction with mycelia extracts of all tested cereal infecting *Fusarium* species was selected for the test development. This antiserum also was therefore chosen because it showed in ELISA variants no cross-reactions with mycelia extracts of other fungus species outside of the genus *Fusarium* and detects in Western blotting experiments specifically glycoprotein bands in *Fusarium* infested wheat grains (Rabenstein, 2002). The plate trapped antigen (PTA)-ELISA was performed in NUNC PolySorb ELISA plates (code:475094) using polyclonal IgG of antiserum PAS Fc 7/2 as follows: A 100 µL antigen extract per well was incubated for 2 h at 37°C (plates were poured out without washing). A 200 µL blocking solution per well was added (1 % nonfat dry milk powder (TM) in PBS), further incubated for 1 h at 37°C, and subsequently washed three times with PBS-Tween 20. A 100 µL per well IgG of PAS Fc 2/7 (conc. 1 µg mL^{-1}) was added in blocking solution and incubated for 1 h at 37°C and washed four times with PBS-Tween. A 100 µL per well alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) (DIANOVA, Hamburg, Germany, code: 111-055-003) diluted 1:2000 in 0.05 *M* Tris-HCl-buffer (pH 8.0) containing 1% TM was added and incubated for 1 h at 37°C, washed four times with PBS-Tween. Finally, 200 µL substrate per well was incubated with p-nitrophenyl phosphate (1 mg mL^{-1} in substrate buffer pH 9.6) for 1 h at room temperature and absorbance was measured at 405 nm with TECAN "Rainbow" ELISA reader (TECAN SLT Lab Instruments, Crailsheim, Germany).

Mycotoxin analysis

Wheat grains were ground into fine flour with a 1-mm sieve and mycotoxins were extracted by weighing out 5 g of each sample in a 100 mL Erlenmeyer flask containing 100 mL double distilled water, covered with parafilm and placed in a rotary shaker (200 rpm) for 5 min. About 1 mL of the suspension was transferred into Eppendorf tubes and centrifuged (14000 rpm) for 5 min. The supernatant was collected and served as stock solution for dilution preparations optimum for analysis.Mycotoxin production in mg kg^{-1} DON and 3-acetyldeoxynivalenol (3-ADON) from ground wheat grain samples was

analysed using RIDASCREEN™ FAST DON (R-biopharm GmbH, Darmstadt, Germany), which is an immunoassay for the quantitative analysis of DON in cereals, malt and feed. The test plates were measured at 405 nm with the aid of a microtiter plate spectrometer (TECAN SLT Lab Instruments, Crailsheim, Germany) and DON content was calculated by using a software package distributed by the manufacturer. Calculation was based on the extinction of five standard solutions in water (0 ppm, 0.222, 0.666, 2 ppm, and 6 ppm) provided by the immunoassay kit per plate. The test cannot differentiate between DON and 3-ADON and has negligibly low, or no cross reactivity to nivalenol and Fusarenon X. Due to high cost of DON-ELISA, only two replicates from each field experiment were analysed.

Statistical analyses

Plot means were used for analysis of variance for each location separately. Residuals were independent and followed a normal distribution for head blight rating, but not for *Fusarium* ExAg content and DON production. The latter two trait values were adjusted to normality by by natural log (ln) and square root transformation, respectively. Error variances were homogeneous across locations according to Bartlett's test (Snedecor & Cochran, 1989). The three environments (year-location combination) were then analysed as a series of random environments according to Cochran & Cox (1957). To allow direct comparison between trait means of different units, coefficient of genotypic variation (cv%) of the respective variance components (σ^2) was calculated according to the formula (σ/mean) x 100. Repeatability estimates were calculated by partitioning the phenotypic variance of spatial replications within one experiment according to the formula $\sigma^2_g /(\sigma^2_g + \sigma^2_e)$ where σ^2_g = genotypic variance and σ^2_e = error variance (Falconer & MacKay, 1996). Estimates of variance components were calculated as described by Snedecor & Cochran (1989). Broad-sense heritabilities (h^2) were estimated on an entry-mean basis (Fehr, 1987) as the ratio of genotypic to phenotypic variance using the formula: $h^2 = \sigma^2_g/(\sigma^2_e/RE + \sigma^2_{ge}/E + \sigma^2_g)$, where σ^2_{ge} = genotype-environment interaction variance, R= number of replicates and E = number of environments. Confidence intervals of heritability were computed according to Knapp & Bridges (1987). All correlations between traits were calculated without the parents. All statistical analyses were computed using the statistical package PLABSTAT (Utz, 2000). The

effects of progeny were considered as fixed whereas the effects of environments and replicates were assumed to be random variables.

Results

Mean fungal exoantigen levels in grain from the untreated plots were both 0.03 units for HOH2001 and HOH2002 and 0.1 units for OLI2002 (data not shown). The latter indicates slight contamination by *G. zeae* or possibly by other *Fusarium* spp. Slight infection was visible in control plots (0.73%). Among progeny, there was a wide range of means for aggressiveness traits; head blight rating and relative plot yield were 4.3 to 48.1%, and 48.4 to 95.9%, respectively. Mean DON production of the progeny was highest in HOH2001 and lowest in HOH2002, although head blight rating, relative plot yield, and ExAg content were similar (Table 1).

Mean head blight rating ranged from 5.03 to 47.75% among 50 progeny (Fig. 1). This range was identical to that of the original 153 progeny. Mean *Fusarium* ExAg content and DON production ranged from 0.26 to 1.41 optical density (OD) units and 4.2 to 43.7 mg kg^{-1}, respectively (Fig. 2). Mean head blight rating and DON production of the parent FG3211 were higher across environments than those of FG24 while *Fusarium* ExAg content of the two parents was similar. All traits exhibited a continuous distribution with performance of the parents not significantly different from each other across environments. DON production in the majority of progeny was higher than the parents. Some progeny significantly exceeded the higher parent value indicating transgressive segregation for higher DON production. Significant genotypic differentiation existed for all traits except for DON/*Fusarium* ExAg ratio when combined across environments (Table 2). Coefficients of variation among progeny were approximately as high as progeny-environment interaction for head blight rating and relative plot yield. Estimate of error was highest for DON/*Fusarium* ExAg ratio. All traits were least affected by replicates. Medium heritabilities were obtained for aggressiveness traits and DON production.

The two aggressiveness traits, head blight rating and relative plot yield, were tightly associated (r = -0.90, P = 0.01, data not shown) as well as *Fusarium* ExAg content and DON production (r = 0.82, P = 0.01) (Fig. 3). Head blight rating was correlated to both DON production and *Fusarium* ExAg content but DON/ExAg ratio did not show any

covariation (Fig. 4). These trait correlations were consistent in HOH2001 and HOH2002 (r = 0.59-0.70), but lower in OLI2002 (r = 0.35-0.59). Coefficients of correlation between relative plot yield and DON production and between relative plot yield and *Fusarium* ExAg content were more similar than for head blight rating (-0.62, -0.55, respectively, P = 0.01). Due to missing genotypic variance, correlation to DON/*Fusarium* ExAg ratio could not be given.

Discussion

Large genetic variation in aggressiveness, fungal colonization, and mycotoxin production has been found in a segregating population of *G. zeae* across field environments. Analysis of progeny allows conclusions on the inheritance of these traits. The parents were not very different from each other although they were selected for differences in a previous study (Miedaner *et al.*, 2000). Obviously, the strong isolate-environment interaction found also in this study prevented this earlier result to be repeated. Despite the phenotypic similarity of the parents, their progeny varied significantly (P = 0.01) indicating the segregation of several genes for each of the traits with additive gene action. Medium heritabilities for the two aggressiveness traits and DON production in the host indicate the importance of the genetic component. *Fusarium* ExAg content in individual environments showed also significant (P = 0.01) genotypic differences in HOH2001 and HOH2002, but not in OLI2002 (data not shown). A possible cause might be the considerable amount of ExAgs detected in the untreated plots in OLI2002 due to contaminating *Fusarium* species although only slight symptoms were visible. Contaminating *Fusarium* spp. also present in the plots inoculated with the progeny could have biased the effect of the inoculated isolates of *G. zeae*. Several studies in Europe revealed that grains infected with *F. graminearum* are also infected by other *Fusarium* species (Schütze *et al*., 1997; Waalwijk *et al*., 2003). In HOH2001 and HOH2002 *Fusarium* ExAgs in the untreated plots were not detectable. High ExAg content in each environment was related to lower plot yield. This was consistent with the finding that levels of fungal biomass measured as ergosterol (ERG) increased as kernel weight decreased (Dowell *et al*., 1999; Danks *et al*., 2001). Several studies have shown that ERG production is closely associated with aggressiveness (Lamper *et al.*, 2000; Mesterhazy, 2002).

All progeny were inoculated on the same susceptible wheat variety, equalizing the effects of host genotype on aggressiveness traits or DON and *Fusarium* ExAg production. All 50 progeny and their parents invaded the heads successfully as shown by disease symptoms, *i.e.*, all progeny were pathogenic despite their large differences in DON production. Similarly, a transgenic isolate of *G. zeae* with a deleted gene for toxin production was still able to successfully infect wheat, rye, and maize, although aggressiveness was reduced (Proctor *et al*., 1995; Desjardins *et al.*, 1996; Harris *et al*., 1999; Bai *et al*., 2001).

DON production was tightly correlated with *Fusarium* ExAg content (Fig. 2) being in agreement with earlier findings (Miedaner *et al*., 2000; Wanyoike *et al*., 2002b). In contrast, Asran & Buchenauer (2003) and Gilbert *et al.* (2001) found no correlation between disease severity and ERG content caused by *G. zeae* in maize seedlings and wheat, respectively.

The ratio of DON/*Fusarium* ExAgs did not vary significantly ($P > 0.1$), *i.e.*, all progeny produced a similar amount of DON relative to their amount of mycelium in the host tissue. Similar results have been reported for 42 isolates of *F. culmorum* using ERG as a measure for fungal biomass (Gang *et al*., 1998). Wanyoike *et al*. (2002b) also found low to moderate correlations only between DON/ERG ratio and head blight rating of 15 isolates of *G. zeae.* It cannot be concluded from such studies what was the cause or the effect. A high DON production of a progeny might be the cause for a fast invasion of host tissue and allow the isolate to produce high amounts of mycelium. Alternatively, the aggressive isolate might speed up invasion because of a third unknown factor consequently produce more DON. Such factors might be other mycotoxins or cell-wall degrading enzymes such as cellulase, xylanase, and pectinase (Balazs & Bagi, 1997; Wanyoike *et al*., 2002a; Schwarz *et al*., 2002). Molecular analyses will provide more insights into these processes in the future. Mitogen-activated protein kinases (MAPKs) have already been identified as important for pathogenicity and development of perithecia in *G. zeae* (Hou *et al*., 2002). Further research should also take into account the early phases of pathogenesis for explaining causes of aggressiveness because DON is already produced after 48 h p.i. (Evans et al., 2000). Doohan *et al.* (1999) reported a high relative *TRI5* (trichothecene gene cluster encoding trichodiene synthase) expression with a minimum amount of GUS activity (β-D-glucuronidase activity) on wheat seedlings infected by a *F. culmorum* GUS transformant G514 (which constitutively

expresses GUS) 10 days after inoculation. At the end of pathogenesis, however, the relationship between the two traits was inverse. In contrast to previous studies, all isolates tested here were progeny from the same cross, thus possessing the same genetic background and mycotoxin profile. This assures that differences in DON or mycelium production were not just caused by isolate-environment interaction, that might play a role when isolates from different geographic origins are tested, or by different profiles of those mycotoxins that have not been analysed. As a consequence, it is essential to consider DON production, fungal colonisation (ExAg content) and DON/ExAg ratio of each isolate in order to establish associations between disease symptoms and fungal characteristics relating to aggressiveness.

Acknowledgements

We thank Mrs. Bianca Schneider and Mrs. Monika Wesermann for the excellent technical assistance in the field and laboratory and the German Academic Exchange Service (DAAD) for the Ph.D. fellowship given to the first author. The second author was partially supported by a grant from the German Federal Ministry for Education and Research (BMBF) (FKZ:03i0606A). We sincerely acknowledge our close cooperation with Dr. Robert L. Bowden and Dr. John F. Leslie of Kansas State University, USA.

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Table 1 Means for head blight rating, relative plot yield, *Fusarium* exoantigen (ExAg) content, deoxynivalenol (DON) production and DON/*Fusarium* ExAg ratio in the moderately susceptible wheat genotype Drifter inoculated with 50 progeny of *Gibberella zeae* cross FG24 x FG3211 and their parents in three field environments

Trait	Isolates	Environment[a]		
		HOH2001	HOH2002	OLI2002
Head blight rating (%)	Progeny	30.01	30.31	22.89
	FG24	13.87	10.22	12.44
	FG3211	14.95	37.21	21.58
Relative plot yield (%)	Progeny	72.65	73.11	57.08
	FG24	96.18	98.68	71.48
	FG3211	80.30	65.80	58.64
Fusarium ExAg content (OD units)[b]	Progeny	0.80	0.75	1.07
	FG24	0.42	0.50	1.00
	FG3211	0.30	0.71	1.12
DON production (mg kg^{-1})	Progeny	27.45	9.69	13.55
	FG24	9.40	3.09	8.29
	FG3211	12.30	12.32	9.24
DON/*Fusarium* ExAg ratio	Progeny	36.41	14.36	13.56
	FG24	23.74	6.87	7.52
	FG3211	25.80	15.56	6.54

[a]HOH = Hohenheim near Stuttgart, OLI = Oberer Lindenhof near Reutlingen. Numbers designate years.
[b]Optical density measured at 405 nm.

Table 2 Coefficients of variation (cv%) for head blight rating, relative plot yield, *Fusarium* exoantigen (ExAg) content, deoxynivalenol (DON) production and DON/*Fusarium* ExAg ratio in the moderately susceptible wheat genotype inoculated with 50 progeny of *Gibberella zeae* cross FG24 x FG3211 averaged across three field environments

Parameter	Head blight rating	Relative plot yield	*Fusarium* ExAg content (transformed)	DON production (transformed)	DON/ *Fusarium* ExAg ratio
Source of variation:					
Environment (E)	14.54*	12.44	9.81^{+}	19.33*	24.61**
Replicate: E	5.37**	7.03**	4.84**	5.78**	-[a]
Progeny (P)	26.65**	12.77**	8.61*	18.35**	4.40
P x E	25.43**	12.97**	13.52**	12.85**	10.83
Pooled Error	18.56	16.23	19.69	14.71	25.91^{+}
Heritability (h^2)	0.72	0.62	0.37	0.79	-[c]
90% C.I. on h^{2b}	0.53-0.82	0.37-0.76	0-0.61	0.65-0.87	-[c]

$^{+}$, *, **Significant at probability levels $P = 0.1$, $P = 0.05$, and 0.01, respectively.

[a]Negative estimate.

[b]Confidence intervals (C.I.) on h^2 were calculated using the method of Knapp & Bridges (1987).

[c]No significant genotypic variance ($P > 0.1$).

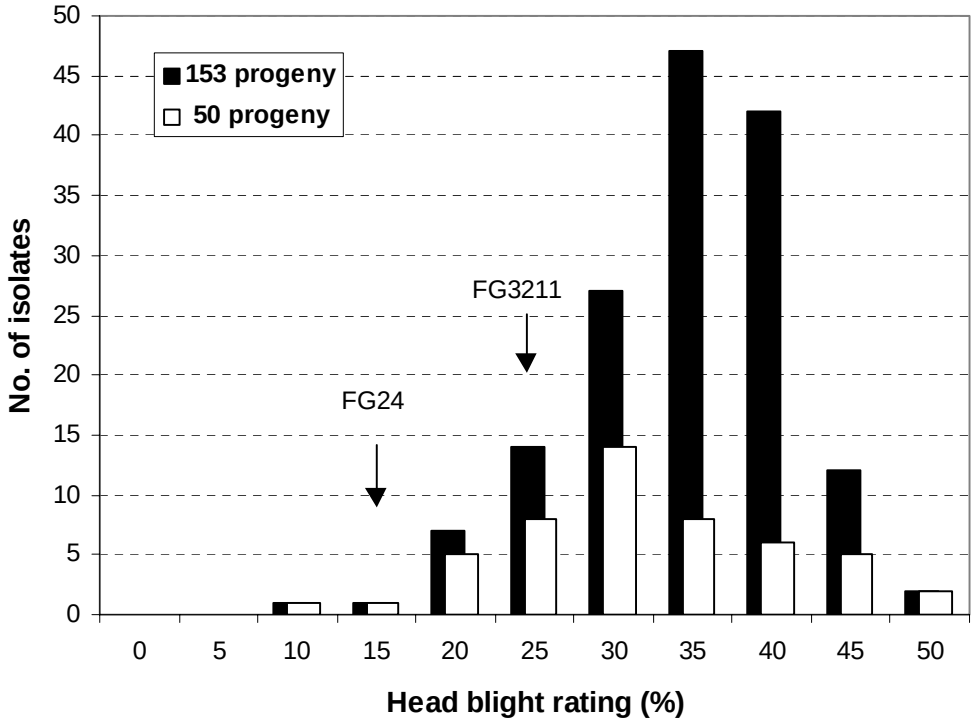

Figure 1 Frequency distribution of head blight rating of all 153 progeny and 50 selected progeny of *Gibberella zeae* cross FG24 x FG3211 combined across three field environments in the moderately susceptible wheat genotype Drifter (untransformed data). Arrows indicate the parents.

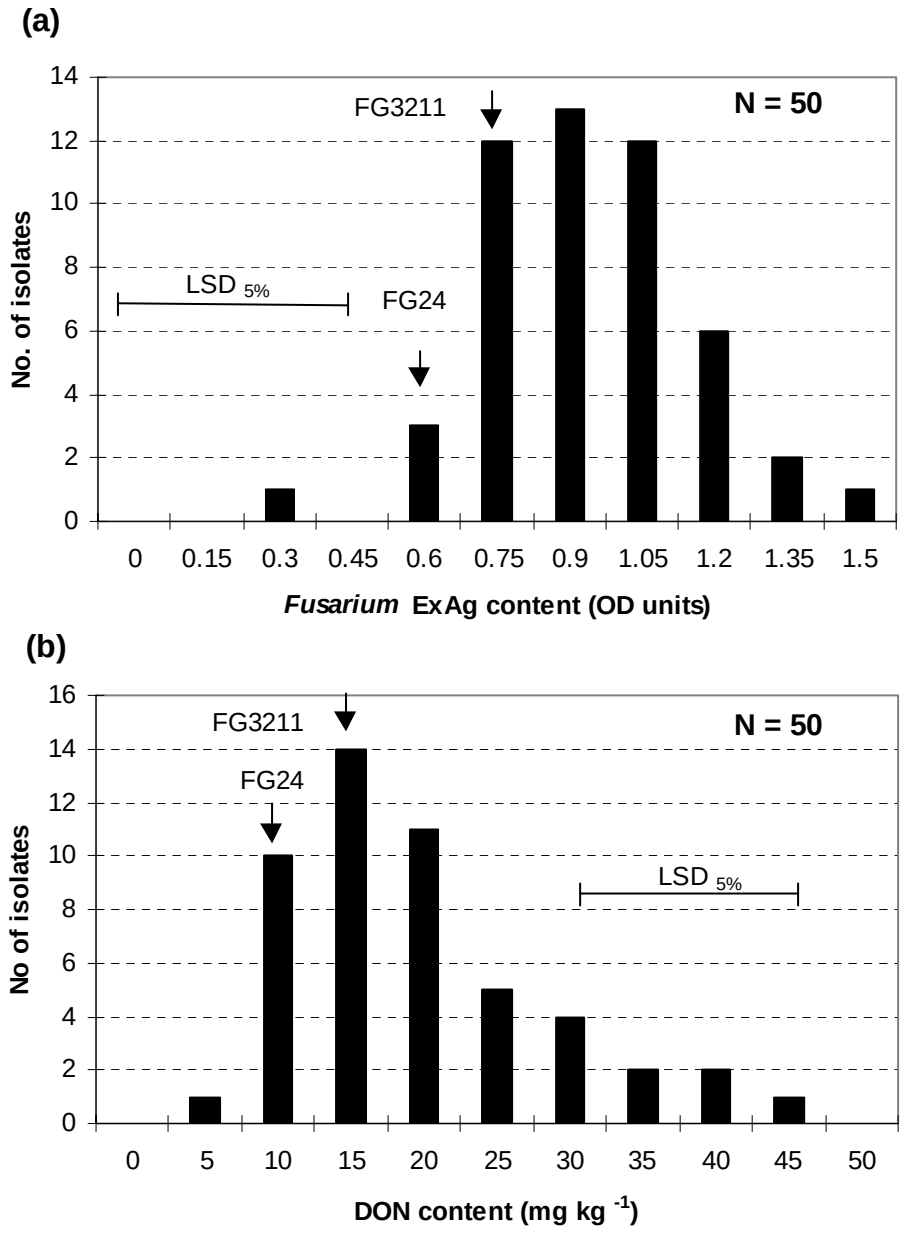

Figure 2 Frequency distribution of (a) *Fusarium* exoantigen (ExAg) content, and (b) deoxynivalenol (DON) production in the moderately susceptible wheat genotype Drifter inoculated with 50 progeny of *Gibberella zeae* cross FG24 x FG3211 combined across three field environments (untransformed data); $LSD_{5\%}$ = least significant difference at probability level $P = 0.05$. Arrows indicate the parents.

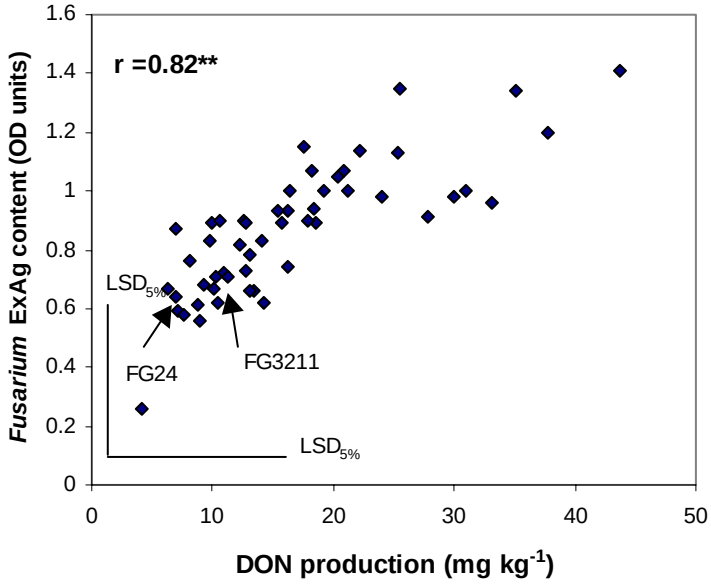

Figure 3 Association between deoxynivalenol (DON) production and *Fusarium* exoantigen (ExAg) content for 50 progeny of *Gibberella zeae* combined across three field environments. (untransformed data); $LSD_{5\%}$ = least significant difference at probability level $P = 0.05$. Arrows indicate the parents.

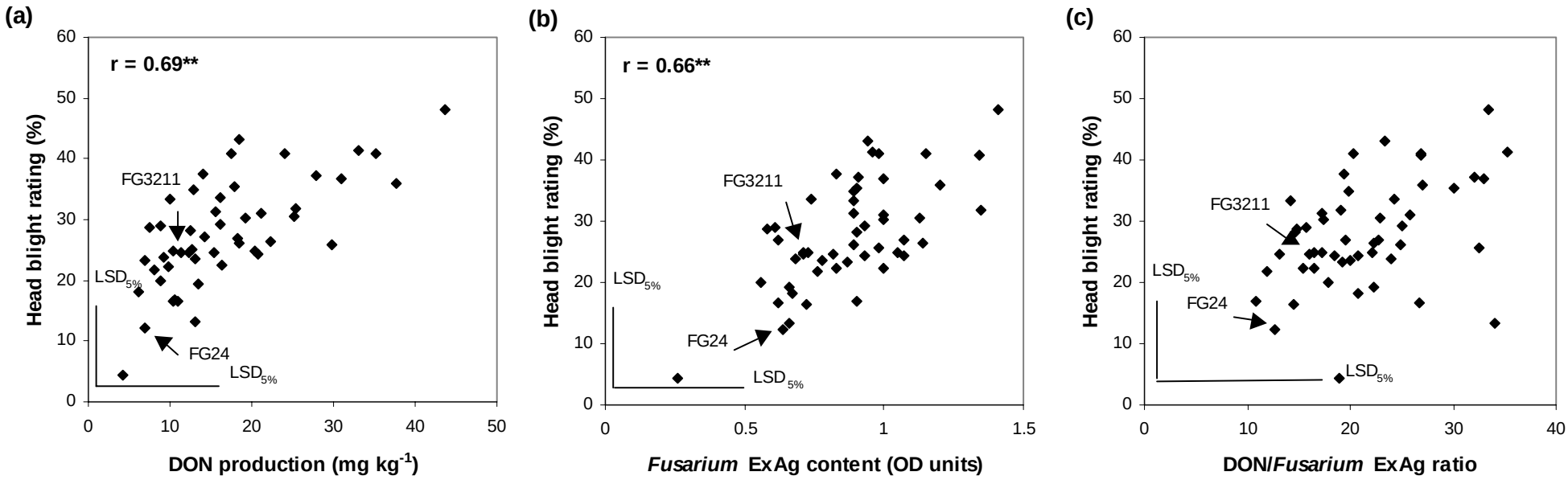

Figure 4 Associations between head blight rating and (a) deoxynivalenol (DON), (b) *Fusarium* exoantigen (ExAg) content, and (c) DON/*Fusarium* ExAg ratio for 50 progeny of *Gibberella zeae* combined across three field environments (untransformed data); $LSD_{5\%}$ = least significant difference at probability level $P = 0.05$. Arrows indicate the parents.

Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz
Journal of Plant Diseases and Protection
110 (6), 554–559, 2003, ISSN 0340-8159
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Short Communication/Kurzmitteilung

Aggressiveness of 42 isolates of *Gibberella zeae (Fusarium graminearum)* in wheat under field and greenhouse conditions

Aggressivität von 42 *Gibberella zeae (Fusarium graminearum)*-Isolaten bei Weizen im Feld und Gewächshaus

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Received 28 July 2003; accepted 14 Oct. 03

Summary

Head blight or scab is a destructive disease caused by *Gibberrella zeae (Fusarium graminearum)* in wheat-growing areas of the world. This study was conducted to determine the correlation between aggressiveness of 42 progeny of the *G. zeae* cross FG24 (Hungary) and FG3211 (Germany) in the greenhouse and across three field environments (location-year combinations). Methods of inoculation in the greenhouse and field were single-spikelet injection and spraying, respectively. Aggressiveness was measured as percentage infected spikelets in the greenhouse and head blight rating in the field (0–100 scale). Mean disease severity was similar in the greenhouse and field (34 % and 30 %, respectively). Significant (P = 0.01) genotypic variation in aggressiveness was found in both test systems with a high heritability (h^2 = 0.90). Correlation of aggressiveness between greenhouse and field was moderate (r = 0.66, P = 0.01), suggesting that aggressiveness was a genetically stable trait. Greenhouse aggressiveness could be a predictive measure of the disease severity caused by the same isolates in the field.

Key words: aggressiveness; field; *Fusarium graminearum; Gibberella zeae;* greenhouse; segregating population; *Triticum aestivum*

Zusammenfassung

Ährenfusariosen sind eine bedeutende Pflanzenkrankheit an Weizen, die durch *Gibberella zeae (Fusarium graminearum)* verursacht wird. Die Korrelation der Aggressivität zwischen den Nachkommenschaften einer Kreuzung von *G. zeae* zwischen FG24 (Ungarn) und FG3211 (Deutschland) wurde im Gewächshaus und an drei Feldumwelten (Ort-Jahr-Kombinationen) untersucht. Die künstliche Inokulation der Weizenpflanzen erfolgte im Gewächshaus mittels Einzelährcheninjektion und im Feld durch Besprühen der Parzellen. Die Aggressivität wurde im Gewächshaus als Prozent befallene Ährchen, im Feld als prozentuale Ährenbonitur erfasst. Der mittlere Befall war im Gewächshaus und Feld ähnlich (34 % bzw. 30 %). In beiden Prüfsystemen wurden signifikante (P = 0.01) genotypische Unterschiede mit einer hohen Heritabilität (h^2 = 0,9) gefunden. Zwischen der Aggressivität der Nachkommenschaften im Gewächshaus und im Feld ergab sich eine mäßig hohe Korrelation (r = 0,66, P = 0,01). Die

Aggressivität ist somit eine genetisch stabile Eigenschaft. Die im Gewächshaus gemessene Aggressivität kann für eine Vorhersage der Befallsstärke derselben Isolate im Feld genutzt werden.

Stichwörter: Aggressivität; Feld; *Fusarium graminearum; Gibberella zeae;* Gewächshaus; spaltende Population; *Triticum aestivum*

1 Introduction

Head blight of wheat caused by *Gibberella zeae* Schw. (Petch), anamorph *Fusarium graminearum* (Schwabe), is a disease of world-wide importance (McMullen et al. 1997) causing a considerable reduction in crop yield and mycotoxin contamination of grains (Marasas et al. 1984). Fungicides are not effective enough in controlling the disease and present cultural practices promote disease severity. Resistance breeding is the most economical and effective form of disease management (Martin and Johnston 1982). Evaluation of resistance requires not only uniform environmental conditions for a reliable genetic differentiation of the host but also genetic stability of pathogen aggressiveness. van Eeuwijk et al. (1995) recommended that any aggressive strain should be useful for screening. However, the aggressiveness of an unknown isolate should be tested before inoculation. Both resistance of wheat to Fusarium head blight and aggressiveness of *G. zeae* are quantitatively inherited (Mesterhazy 1995; Miedaner 1997; Cumagun and Miedaner 2003). For testing wheat genotypes for resistance in the greenhouse, inconsistent results concerning the correlation to field data are reported in the literature. Hall et al. (2001) found a very low correlation between greenhouse and field environment in wheat. In contrast, Bai et al. (2001) reported significant association between proportion of scabbed spikelets between greenhouse and field. In another study, resistance to disease spread was repeatable in greenhouse screening of wheat germplasm and had a good correlation to severity in the field (Hu et al. 1999). Such inconsistency in results is the major reason why most resistance screening is done in the field. A high correlation of aggressiveness between greenhouse and field environment would reduce the costs in carrying out aggressiveness tests for plant breeders, pathologists and biotechnologists. In this study, we compared the aggressiveness of a segregating population of *G. zeae* under greenhouse and field conditions.

2 Materials and methods

2.1 Greenhouse and field tests

A medium susceptible German winter wheat genotype ('Drifter') was used for both greenhouse and field experiments. In the greenhouse, wheat was planted in 13 × 13 cm^2 pots (four plants per pot) with five pots per isolate (= replicates) in September 2002 and vernalized at 2 °C for 7 weeks to simulate field conditions during winter. The plants were then transferred to a nonheated greenhouse until the end of tillering. Shooting was artificially induced in a heated greenhouse cabin (21 °C/19 °C day/night) with 16 h light. Plants were cultivated under these conditions until anthesis. Inoculum was produced on wheat grain medium as previously described (Miedaner et al. 1996). Inoculation with the two parents and 42 progeny of the cross FG24 from Szeged, Hungary × FG3211 from Sersheim, Germany, was done at mid-anthesis on four heads per pot by injecting a suspension of approximately 10 µl with 50,000 conidia/ml of each isolate into the left and right floret of a central spikelet on both sides of the head, i. e., four injections per head with a hypodermic syringe needle (0.50 mm gauge) in a climate chamber (21 °C day/19 °C night and 90–100 % relative humidity). Progeny were pre-selected from a total population of 153 progeny based on normal distribution of aggressiveness across three field environments. Plants were inoculated in two batches in the greenhouse chamber because of differences in date of anthesis of the wheat cultivar. Inoculated pots were covered with a plastic sheet for 48 h in the dark to enhance initial infection by 100 % relative humidity and uncovered for another 48 h with artificial light in the same chamber (~55 % relative humidity). After incubation, plants were transferred to a nontemperature controlled greenhouse to allow symptom development. Conditions were 18–20 °C day/15 °C night at a day length of 16 h from 5.00–21.00 h.

Field experiments were carried out in three environments in Southwestern Germany: Hohenheim near Stuttgart in 2001 and 2002 and Oberer Lindenhof near Reutlingen in 2002. Randomization of plots was according to a complete block design with three replicates. Three-row microplots were used (1.2 m length and 0.625 m width), which were arranged in a chess-cross design, i. e., each plot inoculated with an entry (= progeny or parent) was surrounded by four border plots with triticale to avoid plot-by-plot interference. Wheat was inoculated by spraying 500,000 conidia/ml of 42 progeny of *G. zeae* plus the parents at a rate of ~120 ml m^{-2} at anthesis. For inoculation, a hand sprayer powered by a machine with a standardized air pressure of 3 bar was used. Noninoculated plots served as control.

2.2 Disease assessment

For the greenhouse experiment, the number of infected spikelets/head was recorded at four dates (11, 14, 18, and 23 days after inoculation) and adjusted to the total number of spikelets/heads. The relative number of infected spikelets of the four assessment dates was averaged because the correlations among countings were tight (r = 0.96, P = 0.01). Mean disease severity of each isolate was adjusted to its respective batch mean for standardization of data. For the field experiment, head blight rating reflects the percentage infected spikelets per plot on a linear scale from 0–100. The arithmetic mean of the head blight ratings of four assessment dates was used for further analyses.

2.3 Statistical analyses

In the greenhouse, data analyses were based on pot means, i. e., on the disease severity of four individual heads. Each pot was considered as replicate; thus, 4 × 5 heads per isolate were analyzed. For field data, the progeny or parental mean of the three environments was used. To compare both environments by analysis of variance, the five replicates used in the greenhouse were pooled to three replicates as used in the field experiments. Estimates of variance components were calculated as described by SNEDECOR and COCHRAN (1989). Broad-sense heritabilities (h^2) were estimated on an entry-mean basis (FEHR 1987). Confidence intervals of heritability were computed according to KNAPP and BRIDGES (1987). Repeatability estimates were calculated by partitioning the phenotypic variance of spatial replications within one experiment according to the formula $\sigma^2_p / (\sigma^2_p + \sigma^2)$ (FALCONER 1989) where σ^2_p = progeny variance and σ^2 = error variance. All statistical analyses were computed using the statistical package PLABSTAT (UTZ 2000). The effects of replicates and environments were assumed to be random variables.

3 Results and discussion

Quantitative-genetic variation of aggressiveness of 42 progeny of *G. zeae* was observed in the greenhouse as shown by continuous distribution of the trait (Fig. 1). The aggressiveness of the parents did not differ significantly for both environments. Estimates of repeatability for the greenhouse and field tests were 0.4–0.5 and 0.6–0.8, respectively. Progeny exceeded the most aggressive parent, indicating transgressive segregation of the population. Although the method of inoculation and disease assessment differed in the greenhouse and field, disease severity was similar with 33.9 % and 30.1 %, respectively. Almost no background infection was observed in the field as shown by a minimal mean head blight rating of 0.87 % in the noninoculated plots across three environments. This condition should allow a good comparison between field and greenhouse aggressiveness. Genotypic variation of aggressiveness was highly significant with progeny being the most important source of variation accounting for about half of the total variation (Table 1). The method-progeny interaction, although significant, accounted for 12 % of the total variance only. Accordingly, the heritability of aggressiveness was high (h^2 = 0.90), suggesting that a substantial proportion of the observed phenotypic variation was caused by genetic effects.

Greenhouse is considered a highly artificial environment. Wheat plants grown in the field are normally more vigorous compared to those grown in a greenhouse. Evaluation of aggressiveness in the greenhouse, however, can reduce nongenetic variation because the environment can be controlled to a

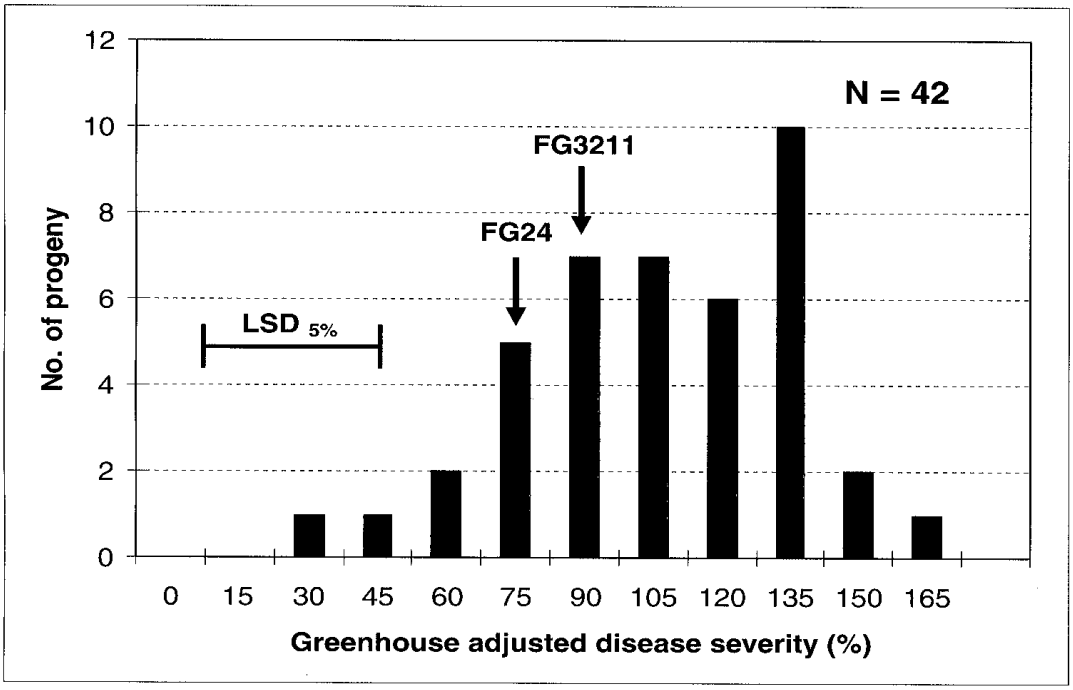

Fig. 1. Frequency distribution of disease severity in the greenhouse adjusted to the respective batch mean for 42 progeny of the *Gibberella zeae* cross FG24 x FG3211 and their parents (arrows) on winter wheat cv. 'Drifter'. $LSD_{5\ \%}$ = least significant difference at probability level P = 0.05.

Table 1. Variance components estimates and heritability of head blight rating for 42 progeny of the *Gibberella zeae* cross FG24 x FG3211 on winter wheat cv. 'Drifter' for one greenhouse and across three field environments

Parameter	Variance component estimate
Source of variation:	
Method of inoculation (M)	4.95
Replicate (R): M	5.73**
Progeny (P)	64.92**
M x P	16.07**
Pooled Error	40.74
Heritability (h^2)	0.90
90 % C. I. on h^2 [a]	0.84–0.94

[a] Confidence interval (C.I.) on h^2 were calculated using the method of KNAPP and BRIDGES (1987).
** Significant at probability level was P = 0.01.

greater extent than in the field. Moreover, single-spikelet injection that measures spread of scab within the spike is a relatively stable method and less affected by the environment (SCHROEDER and CHRISTENSEN 1963). This method was not feasible in our field experiments due to the high amount of labour. The greenhouse environment provides a condition, which favours early and fast Fusarium head blight development in a wheat spike. In the field, in contrast, infection was under natural weather conditions and disease severity is likely to be affected by temperature and humidity. Despite these environmental differences, we found a rather high correlation of aggressiveness between greenhouse and field experiments (r = 0.66, P = 0.01) (Fig. 2). Coefficients of correlation between greenhouse and individual field environments were 0.42, 0.61, and 0.49 for Hohenheim 2001, Hohenheim 2002, and Oberer Lindenhof

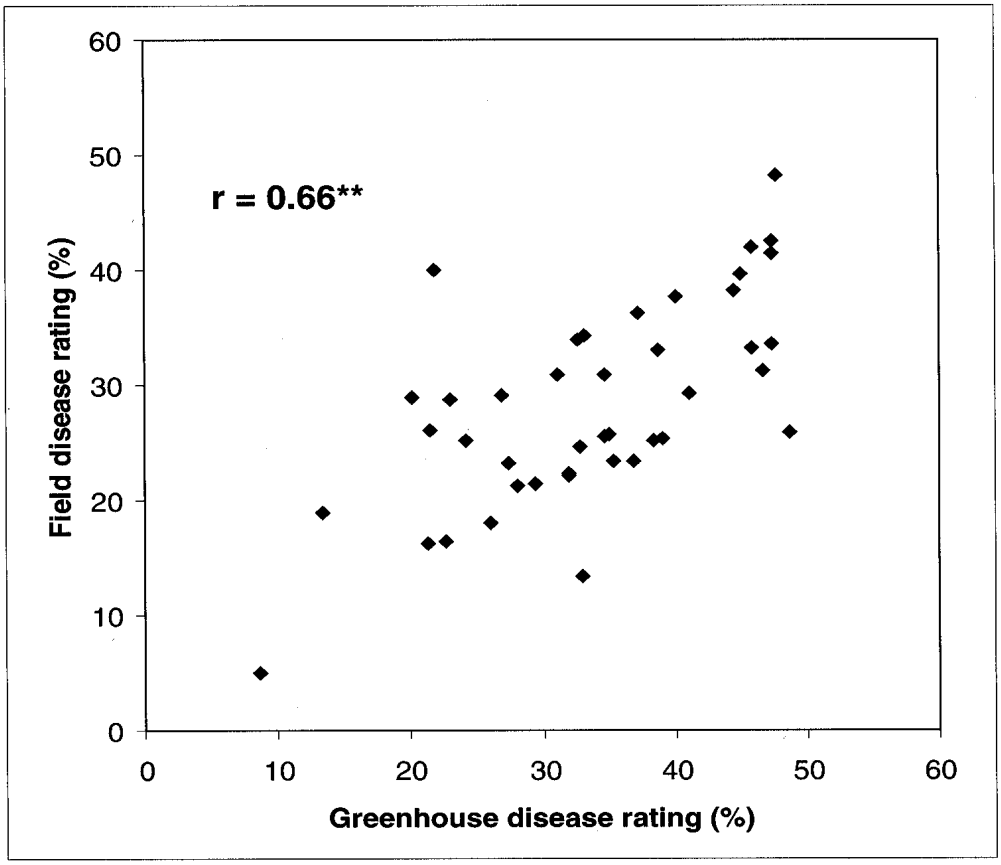

Fig. 2. Correlation of aggressiveness between greenhouse and combined across three field environments for 42 progeny of the *Gibberella zeae* cross FG24 × FG3211.

2002, respectively (P = 0.01). In the greenhouse inoculation, the spores were injected directly into the spikelet of a head, whereas in field inoculation, the fungus had first to invade the wheat head until spreading could occur (KANG and BUCHENAUER 2000). The correlation between two modes of entry shows that the spreading within the head seems to be the major component of aggressiveness. Based on our data, aggressiveness as determined from the greenhouse inoculation could be a reliable estimate of the aggressiveness in the field. The use of a spring wheat in the greenhouse would further accelerate the test.

Acknowledgements

The work was supported by a Ph. D. fellowship from the German Academic Exchange Service (DAAD), Bonn, for the first author.

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7 General Discussion

7.1 Methods and limitations of the study

For genetic studies in fungi, making crosses is a basic approach to generate populations segregating for traits important for fitness and survival of the organism and analyse the genetic basis of phenotypic differences of these traits. This technique is a prerequisite to phenotypic and molecular analyses. The genus *Gibberella* exhibits two reproductive strategies which are needed for sexual reproduction: heterothallic (self-sterile) and homothallic (self-fertile). Heterothallism in some *Gibberella* species like *G. pulicaris* and *G. fujikuroi* facilitates genetic analysis; therefore, the regulation mechanism has been easily analysed by crossing two different mating types (Phinney et al., 1967; Desjardins and Beremand, 1987). In contrast, *G. zeae* is homothallic, vegetatively incompatible, and lacks a parasexual cycle. These characteristics make it difficult and laborious to carry out genetic analysis by crosses in this fungus because of the need to use marked strains to identify heterozygous perithecia. Based on the literature, the frequency of heterozygous perithecia of *G. zeae* can be as low as 0 to 35% (Bowden and Leslie, 1999) and 0 to 21% (Desjardins et al., 2000) in those genomic regions where the *nit* loci are located. To circumvent this barrier, Lee et al. (2001) used drug resistance genes and *MAT* (mating type) gene manipulation to obtain sexual recombinants of *G. zeae*. By targeted manipulation of *MAT*, there is a complete conversion of the fungus from homothallic to heterothallic (Lee et al., 2003). In the present work, nitrate nonutilizing (*nit*) mutants of the parents have been used as suitable markers to distinguish homozygous from heterozygous perithecia, but the disadvantage of this technique is the occurrence of segregation distortion, i.e., markers deviate significantly from the Mendelian ratio. For mapping QTLs of important fungal traits like pathogenicity, aggressiveness, and mycotoxin production, two populations with the following parent crosses were used: (1) pathogenic, high DON-producing Z-3639 from Kansas and nonpathogenic, low NIV-producing R-5470 from Japan belonging to lineage 7 and 6, respectively (O'Donnell et al., 2000; Jurgenson et al., 2000); and (2) two medium DON-producing FG24 from Szeged, Hungary and FG3211 from Sersheim, Germany, both aggressive lineage 7 isolates. Pathogenicity, as defined in our study, is the ability to cause disease while aggressiveness refers to the quantity of disease induced by a pathogenic isolate on a susceptible host in a system in which the isolates do not interact differentially with host cultivars (Vanderplank,

1968). Disease severity of the first population was recorded in the greenhouse for two years and aggressiveness traits (head blight rating and relative plot yield), *Fusarium* exoantigen (ExAg) content (for 50 progeny) and deoxynivalenol (DON) production of the second population in three field environments.

Lineage 6 (R-5470) has not yet been reported in Europe (O'Donnell et al., 2000). Because of quarantine and plant health reasons, we have not tested the aggressiveness of the first population consisting of 99 progeny in German fields. If it were so, we may have found other aggressiveness QTLs in the field that were perhaps masked by controlled environment variables in the greenhouse. Correlation of aggressiveness of the second population between field and greenhouse, however, was moderate to high. We have not mapped QTL for mycotoxin production in this particular cross because data on the toxin produced of all progeny were not measured, but their *in vitro* amounts were treated qualitatively, i.e., as low and high DON or NIV content (Jurgenson et al., 2002).

An unusual character of the first cross is the nonpathogenic reaction of the Japanese parent R-5470. Based on previous studies, nonpathogenic field isolates of *G. zeae* are rarely observed (Mesterhazy, 1981; Miedaner et al., 2000). We used a highly susceptible wheat genotype, an extremely disease-favorable environment, and delivered a relatively heavy inoculum load directly through the glumes into four central florets per head, but even under these very favorable conditions for disease development, nonpathogenic strains only rarely could spread beyond the inoculated spikelets. There was only one case in the literature in which *F. graminearum* cultures were almost nonpathogenic in barley but these isolates were old cultures dating over 50 years in storage (Takeda and Kanatani, 1991). Another hypothesis could be that R-5470 was not representative for the gene pool of *G. zeae* due to laboratory mutation (Bowden, personal communication). This could happen because genetic instability in culture is a common phenomenon in *Fusarium* species (Nelson et al, 1981). Besides that, we do not have any information from the collector, the late Dr. Paul E. Nelson, (Department of Plant Pathology, Pennsylvania State University, University Park, PA) on cultural characters or pathogenicity of R-5470 when it was originally isolated.

Our original goal, aside from mapping aggressiveness in a population of a widely divergent cross of *G. zeae*, was to detect QTLs for aggressiveness and mycotoxin production in a second cross between two European strains. These results should have a higher impact on what is happening in natural populations and verify whether the data from the first cross are representative. Furthermore, this cross between two DON-producing aggressive parents of the same lineage may provide a better view of elucidating the genetic basis of aggressiveness.

Two problems became evident during this study. Firstly, FG24 x FG3211 population was not polymorphic enough to generate a genetic map of the fungus within a reliable time frame. The rate of polymorphism between the interlineage parents Z-3639 and R-5470 was about three to four times greater than between the intralineage parents FG24 and FG3211 using AFLP and RAPD markers. Secondly, about one third of the 46 polymorphic markers had distorted segregation ratios. Consequently, QTL mapping of aggressiveness and DON in the FG24 x and FG3211 population was not initiated.

7.2 Genetic basis of pathogenicity and aggressiveness

Pathogenicity, based on the definition given, connotes a qualitative aspect of the trait, i.e. either a pathogen or not. To our knowledge, studies on the inheritance of pathogenicity and/or nonpathogenicity are unavailable. Aggressiveness, as most often referred to in the literature as pathogenicity, is often described as a polygenic trait (Blanch et al., 1981; Hawthorne et al.,1994; Wroth, 1998). Influence on the rate of evolution of pathogen aggressiveness will depend to a large extent on how aggressiveness is genetically controlled. Mostly, genetic studies of aggressiveness by *Fusarium* species in wheat are lacking and data on quantitative inheritance of these traits are based on collection of field isolates (Miedaner et al., 1996; Miedaner, 1997). Segregating populations generated by inducing sexual reproduction in the lab are more useful for this purpose than a collection of pathogen isolates representing a wide range in aggressiveness. Gene disruption experiments have contributed greatly to our understanding on the role of mycotoxins on aggressiveness, but the technique limits the number of isolates for testing.

QTL analysis of Z-3639 x R-5470 population mapped pathogenicity and aggressiveness on different linkage groups and thus confirmed the genetic distinction between the two traits. A consistent bimodal distribution of pathogenicity and nonpathogenicity in the cross (61:38 pathogenicity:nonpathogenicity) for two years, confirms the high heritability obtained and provides a clear evidence of a single major gene segregating for this trait, although segregation ratio was distorted due to putative chromosomal rearrangement in the genome and the use of the *nit* marker technique in crossing the strains (Jurgenson et al., 2002). Designated as *PATH1*, this qualitative pathogenicity locus was mapped on linkage group IV that is located near loci *PIG1* (red pigmentation), *PER1* (perithecial production) and *TOX1* (toxin level). Recent analyses indicate that at least two of these traits are controlled by *MAP* kinase genes connected to pathogenicity (Hou et al, 2002; Urban et al., 2003). *PIG1*

accounted for the greatest portion of the variation among the pathogenic and nonpathogenic progeny.

The two segregating populations - FG24 x FG3211 and Z-3639 x R-5470 **(**within the pathogenic progeny) - exhibited quantitative inheritance of aggressiveness traits. This implies that more than one gene was involved in the expression of these traits. On the contrary, only two QTLs were mapped by simple interval mapping in the Z-3639 x R-5470 population. Not even composite interval mapping could increase the number of QTLs with appreciable effects. Both aggressiveness QTLs among the pathogenic progeny were mapped on linkage group I. We presume that there was only one locus because two QTLs were reduced into one locus by composite interval mapping. This single-gene hypothesis could be explained from an "receptor–elicitor" perspective. The nonpathogenic R-5470 may encode an elicitor that binds to the host receptor which results in no disease reaction. In the case of Z-3639, no recognition takes place and therefore disease occurs. This could explain the monogenic inheritance of pathogenicity in this cross. Alternatively, the nonpathogenic isolate may have had a knock-out mutation at the tip of linkage group IV that took out genes responsible for host colonization, e.g. extracellular enzymes (Bowden, personal communication). Besides, the ability of the isolate to penetrate the host could not be measured as this factor was ruled out by single-spikelet inoculation with a syringe. All pathogenic isolates inherited the pathogenic allele from the Kansas isolate. Even the nonpathogenic R-5470, however, contributed to the aggressiveness of the pathogenic isolates. R-5470 itself could not cause infection due to missing pathogenicity but its alleles for aggressiveness were expressed among the recombinant progeny.

An alternative hypothesis in explaining a single locus relies primarily on the low number of progeny (N =61). Hence, the power of detecting more QTLs was low especially if the rest of the putative QTLs had relatively small effects. In a model study with ten simulated QTLs and a high heritability (0.90), Beavis (1998) demonstrated that only three to seven QTLs could be detected within a population of about 100 progeny. It should be noted that all assumptions underlying a single locus for aggressiveness are valid for Z-3639 x R-5470 cross only. Additionally, some aggressiveness loci might not differ between the parents of this cross and, therefore, did not contribute to segregation.

Continuous distribution of aggressiveness traits does not necessarily imply polygenic inheritance. It could also be under mono- or digenic control with a large effect of the environment (Allard, 1960). Heritability estimate in the greenhouse experiment was 0.8, implying that genotype-environment interaction was influencing the trait. Moreover, the

frequency distribution of the pathogenic subpopulation was continuously distributed, but deviated from normality indicating that only a few loci were segregating for aggressiveness in this cross. The FG24 x FG3211 population was characterized by high isolate-environment interaction due mainly to the manner in which the 153 progeny were tested. Progeny were inoculated in three batches at three consecutive days to reduce the amount of work to a manageable level. Microclimatic conditions in subsequent days of inoculation, however, might have been critical in influencing disease severity (Reinbrecht, 2002). Consequently, this strategy of inoculating large number of progeny most probably enhanced nongenetic effects as shown by the high importance of batch-environment and batch-replicate interaction. Reproducibility of inoculation within one batch (= inoculation day) was demonstrated by the medium to high repeatability estimates. Despite different environmental conditions, aggressiveness in this cross might be under different genetic control than that of the first cross. Pathogenicity seems to be less affected by the environment than aggressiveness of the Z-3639 x R-5470 cross.

Quantitative inheritance of aggressiveness has been observed in other fungal pathosystems. A cross between a weakly pathogenic and a highly pathogenic isolate of *Gaeumannomyces graminis* exhibited continuous range of aggressiveness in the greenhouse with no evidence of major gene nor non-additive effects (Blanch et al., 1981). Analysis of progeny from a very high and very low aggressive parents of *Nectria haematococca* that differed also in colony pigmentation showed quantitative genetic control of aggressiveness (Hawthorne et al., 1997). Six to twelve effective factors (or QTLs) were found, owing most likely to the high number of progeny analysed (N=800). These two pathosystems have in common with *G. zeae* that pigmentation allele explained the highest proportion of phenotypic variation in aggressiveness.

For comparison to the field, one third of the progeny (N=42) of the FG24 x FG3211 population was tested for their aggressiveness in the greenhouse. Aggressiveness in the two environments was moderately correlated when calculated on the basis of mean aggressiveness of three field environments. Greenhouse aggressiveness could be a predictive measure of the disease severity caused by the same isolates in the field; thus could reduce the costs for aggressiveness tests. Although methods of inoculation were different, i.e., injection for greenhouse and spray for field, disease spreading within the head seems to be the major component of aggressiveness. In contrast, the correlation between the two methods was low to medium using 20 wheat genotypes with the same isolate of *F. culmorum* in a multienvironment trial (Miedaner et al., 2003). Two of the components in FHB resistance,

namely initial infection and spread of symptoms within a spike, seem to be not fully and genetically related in wheat (Buerstmayr et al., 2003).

7.3 Genetic basis of trichothecene type and production

Biosynthetic pathway genes of trichothecene production of *Fusarium* are closely linked and constitute a gene cluster. The expression of these genes is under complex regulation (Tag et al., 2001). So far, 11 genes involved in trichothecene production have been identified in *F. sporotrichioides* and *F. graminearum* and 10 of the genes are clustered (Brown et al., 2001). These clusters are presumably conservative and narrow; thus minimal or no genetic recombination during meiosis could take place. One QTL in this genomic region might correspond to several genes that are located in close vicinity. One locus for aggressiveness was located in our population near *TRI5*, part of the trichothecene gene cluster that encodes a trichothecene synthase, on linkage group I. *TRI13*, the gene that determines whether DON or NIV will be produced is in the same cluster (Lee et al., 2002). On the basis of this knowledge, we presume that *TRI5* could be a proxy for *TRI13* as the QTL that peaked on this marker clearly distinguished the aggressiveness of the two mycotoxin chemotypes. On average, DON-producing isolates were twice as aggressive as the NIV-producing isolates. The type of mycotoxin produced, either DON or NIV was inherited in a Mendelian way (Jurgenson et al., 2002).The amount of toxin produced was governed by *TOX1* on linkage group IV. *TOX1* for the Z-3639 x R-5470 population also segregated as a single Mendelian character (Jurgenson et al., 2002). The localization of this gene is in contrast with the reported regulatory gene *TRI10* controlling toxin production within the trichothecene cluster on linkage group I (Tag et al., 2001). *TOX1* should be further studied because of its large effect on toxin biosynthesis (Jurgenson et al., 2002). The putative qualitative segregation of toxin level in the Z-3639 x R-5470 population does not contradict to the quantitative inheritance of DON production observed in the FG24 x FG3211 population because toxin levels in the first cross were assessed into two discrete classes only, i.e., high DON and high NIV which were associated to specific AFLP fragment sizes 1700 bp and 2100 bp, respectively. Horizontal transfer of DNA has been suggested to be a driving force in the formation of gene clusters in fungi and bacteria (Rosewich and Kistler, 2000). A number of genes related to pathogenicity are also clustered in plant pathogenic fungi. In *Aspergillus*, mycotoxin genetic clusters might serve as survival mechanism prior to harvest under adverse ecological conditions (Sidhu, 2002). Several studies showed that DON is synthesized during

pathogenesis by *G. zeae* a couple of hours after inoculation (Evans et al., 2000, Savard et al., 2000, Bai et al., 2002). Miller et al. (1983) demonstrated that stressful conditions favor trichothecene production by *G. zeae*.

The effect of environment on DON production of the progeny was very evident. This is consistent with the finding that trichothecene biosynthesis could be highly affected by environmental conditions such as temperature, substrate, and moisture content (Greenhalgh et al., 1983; Mesterhazy, 2002). Isolates of *G. zeae* highly varied in their DON production in a reproducible manner under optimal growth conditions (Mirocha et al., 1989; Gang et al., 1998; Walker et al., 2001). In our study, DON had a lower environment stability than aggressiveness.

Fumonisins of *G. fujikuroi* are closely related to trichothecenes of *G. zeae* in their role as a putative aggressiveness factor in seedling blight of maize as revealed by genetic and molecular analyses (Desjardins et al., 1996). Although the genetic map of *G. fujikuroi* is already existing (Xu and Leslie, 1996), QTL mapping has not been initiated but the ability to produce fumonisins, particularly fumonisin B_1, is inherited as a single gene or group of closely linked genes (Desjardins et al., 1992). Later studies confirmed that these single gene differences could represent gene clusters in *G. fujikuroi* (Desjardins et al., 1996). Similar inheritance in the synthesis of naphthazarine toxins is reported for *N. haematococca* (Marasas et al., 1984).

7.4 Association between aggressiveness and mycotoxin production

High correlations between aggressiveness traits and DON production *in planta* of *G. zeae* across three field environments suggest that the two traits were controlled by the same QTL. The strength of relationship between aggressiveness and DON production was higher from isolates of a segregating population than from a collection of field isolates. Investigating the role of toxins as aggressiveness factors has been a milestone in fungal genetic analysis (Proctor et al., 1995; Harris et al., 1999). A disrupted *TRI5* gene from a nonproducing trichothecene mutants of *G. zeae* initially tested in a growth chamber caused less disease compared to the trichothecene-producing wild type in wheat. These findings favoured the view that trichothecene production is an aggressiveness factor and not an essential pathogenicity factor. Difference in the aggressiveness of the wild type and mutant deficient strain, however, was not significant in all wheat cultivars. Low levels of disease caused by a nonproducing trichothecene mutants suggests that there are other factors that contribute to the

disease caused by *G. zeae.* Aside from trichothecenes, *G. zeae* produces enzymes, hormones and other metabolites, such as zearalenone and fusarins which might play a role in pathogenesis. Wanyoike et al. (2002) demonstrated that reduced gold labelling densities present in the infected host cells suggest that these polysaccharide degrading enzymes could be important pathogenicity factors of *G. zeae* during infection of wheat spikes or components of aggressiveness. Enzymes such as cellullose, polygalacturonase, β-glucanase, xylanase, and proteinases are suspected to be involved in the grain colonization and pathogenesis (Balazs and Bagi, 1997; Schwarz et al., 2002) whose function in cell wall degradation is a major characteristic of necrotropic pathogens (Brasier, 1987). There are indeed many possible components of aggressiveness and therefore it is not surprising that aggressiveness of *G. zeae* or *F. culmorum* has a quantitative-genetic basis (Miedaner et al., 1996; Miedaner et al., 2000). DON is reported to aid in fungal colonization (Snijders and Krechting, 1992; Nicholson et al., 1998). We found a rather high correlation between DON production and *Fusarium* exoantigen (ExAg) content of 50 progeny of the *G. zeae* cross FG24 x FG3211, suggesting the feasibility of using ExAg content as an indirect measure for the presence of DON in the grain. Although the genetic make-up of the population originated from one cross, ExAg has low heritability, at least as reported here, partly due to high progeny by environment interaction and error. If QTL for *Fusarium* ExAg content could only be mapped, we expect that it should be residing near or within the aggressiveness QTL. Our study showed that all isolates produced the same amount of DON relative to their ExAg content. The respective ratio had no significant genotypic variation ($P > 0.1$), although the single components, DON and ExAg content, varied. This also demonstrated that DON itself cannot be the only component of aggressiveness. Based on correlation and QTL studies, however, it cannot be decided whether two factors have a causative or merely correlative association. Furthermore, close linkage between two loci, e.g. as found for *PATH1* and *TOX1* or one major locus for aggressiveness and *TRI5* in our studies provide no hint for a causal association. This question can only be solved if such linkage disequilibria are formed consistently on several different crossing populations or if the underlying genes are cloned and identified.

7.5 Significance for population structure of *G. zeae* and breeding for resistance

Miedaner (1997) proposed that sudden increase in aggressiveness in the presence of FHB resistant varieties is unlikely considering that *G. zeae*, undergoes saprophytic phase, is not highly specialized as a pathogen, and because no races could be found that specifically

infect host genotypes. Host resistance is therefore likely to be durable. In view of the rather simple inheritance of aggressiveness however, aggressiveness level of natural populations of *G. zeae* might gradually increase if oligogenically inherited host resistances are widely grown. For example, the Chinese resistance source Sumai 3, that is used worldwide in breeding programs because of its outstanding resistance performance, has been shown to harbor only two QTLs explaining about 50% of the total variance (Buerstmayr et al., 2003). Kolmer and Leonard (1986) already demonstrated the gradual erosion of quantitative resistance in corn by a selection for virulence of *Cochliobolus heterostrophus* in the lab. This might also be true for other pathosystems (McDonald and Linde, 2002). The high propagule number of *G. zeae* produced in disease epidemics reinforces the role of mutation as an evolutionary force. Spontaneous mutation frequency per aggressiveness gene in fungi is estimated at 1×10^{-6} on average (Fincham et al., 1979). *G. zeae* has large population size and possesses a mixed reproduction system with both sexual and asexual reproduction. Asexual reproduction occurs more frequently than sexual reproduction. Cultural practices such as maize-wheat rotation would induce sexual reproduction, allowing at least one recombination per year, and dramatically increase asexual spore production. At the current situation, we consider *G. zeae* a medium-risk pathogen because gene flow plays a minor role only (McDonald and Linde, 2002). As a soil-borne pathogen, long–distant transport is limited and can only occur by seedborne inoculum. Nevertheless, the large genetic variation of *G. zeae* in individual field populations (Bowden and Leslie, 1992; Miedaner and Schilling, 1996) implies that sexual recombination and mutation should play a role in the pathogen evolution. Nothing is known about the time frame and relative importance of such processes in the field. Therefore, erosion of host resistance by the pathogen cannot be ignored. Owing to the presence of transgressive segregation observed within a European cross of *G. zeae*, we present evidence that aggressiveness could increase in the long run and may lead to a gradual unspecific adaptation in the progeny of crosses within lineage 7. Zhan et al. (2002) found greater stability, higher genotype diversity and smaller selection coefficients of *Mycosphaerella graminicola* isolates collected from the moderately resistant wheat cultivar compared to a susceptible one. This clearly shows that host genotype may have an impact on the dynamics of pathogen populations even in quantitative host-pathogen-interactions. Resistance to FHB could erode over longer periods of time when highly resistant host varieties are grown on large acreages. A resistant genotype to FHB was found highly stable in 16 environments (Mesterhazy, 1995). The experiment, however, was conducted in small plots.

There are two challenges faced by wheat breeders selecting for high FHB resistance: (1) oligo-to polygenic inheritance of resistance to *G. zeae*; and (2) impact of environment on disease. Solutions to these challenges involves investigation of both pathogen and host components. The counterpart of quantitative resistance of the host is aggressiveness of the pathogen. The study of pathogen fitness traits including mycotoxin type and production should help elucidate host resistance mechanisms and environmental factors that affect disease development. Several studies have concentrated on quantitative resistance of the host with very limited work on the quantitative aggressiveness of *G. zeae*. The role of toxins as aggressiveness factors should be supported by plant genetic analysis. If toxin production increases pathogen aggressiveness, it follows that increase in host resistance to the toxin should decrease aggressiveness in populations of *G. zeae* (Snijders and Krechting, 1992). Due to our specific cross Z-3639 x R-5470, we found, by QTL analysis, a genetic difference between pathogenicity and aggressiveness. Pathogenicity and aggressiveness are confusing terms for students of plant pathology. Different authors use different terms to describe the same concept or use the same terms for different biological concepts as had been cited before. The term aggressiveness is not so much used in plant pathology. For example, the British Society of Plant Pathology rejected the term aggressiveness and considered it to be synonymous with pathogenicity (Holliday, 1989). The distinction between the two terminologies by genetic mapping better defines these fitness traits and should be adapted by plant pathologists.

7.6 Research needs and outlook

We found that Kansas parent Z-3639 closely resembled the two European parents from wheat in aggressiveness and DON production but was genetically separated from the Japanese parent R-5470 as shown by AFLP and RAPD analyses. Genetic similarity of the European parents correlated well with the findings of O'Donnell et al. (2000) on lineage grouping and Carter et al. (2002) on the basis of three RAPD profile groups, in which isolates from the USA and North-West Europe formed a single group C. In view of these results, our major concern is whether it would be worthwhile to continue mapping the FG24 x FG3211 population. As an initial strategy, we recommend to analyse the polymorphism of a larger collection of *G. zeae* isolates belonging to lineage 7 by AFLP and RAPD markers. If we find substantial polymorphism within lineage 7, it was by chance that we had selected low polymorphic parents. If not, mapping would still be the direction of this research. In a wheat -

agroecosystem, only isolates of lineage 7 would most likely be coming together by trade in Europe. In principle, mapping an intralineage cross of *G. zeae* is possible when the parents are polymorphic enough (Bowden et al., 2002; Gale and Kistler, personal communication). To avoid segregation distortion when using *nit* marker, a deletion in the *MAT2* gene should be used to induce heterothallism (Bowden et al., 2002).

Crosses of other lineages with more progeny might assist in detecting other QTLs that have lower effects and perhaps map elsewhere in the genome. To assess accurately the effect of aggressiveness factors other than toxin type and remove toxin production from consideration as a pathogenicity factor, an ideal cross for analysis would be between strains that produce similar levels of either DON or NIV but differ in the level of disease severity. This phenotype character is, however, difficult to find because of the correlation between aggressiveness and DON production. Host-isolate interactions have not been reported (Van Euwijk et al., 1995) due perhaps to much concentrated efforts on host studies but not on the genetics of the pathogen. Now that a QTL for aggressiveness has been found, it is possible to look more deeply into cultivar-isolate interactions, e.g. by mapping resistance in a segregating wheat population that is inoculated with different isolates. In the barley-leaf rust pathosystem, Qi et al. (1998) observed race-specificity of some quantitative resistance loci by comparing the respective QTLs when two different rust isolates were inoculated. But QTL analysis is just a starting point in genetic studies. Research should gradually move from initial chromosomal location of QTLs towards gene identification. After a wide genome scan, fine mapping of specific chromosome regions using the markers linked to QTLs of interest, would be the next step. Molecular tools allow QTLs to be isolated, cloned by recombinant DNA technology, and used for functional and evolutionary studies.

Genomics is the latest research trend in biotechnology that analyses the whole genome of an organism. Partial genome sequence information and expressed sequence tag (EST) collections of *G. zeae* have become available (Trail et al., 2003). These data will speed up gene identification involved in host–pathogen interaction and provide new insights into the evolution of fungal parasitism (Leach et al., 2003). The trend of *G. zeae* genetic research - from AFLP map to EST – may lead to a construction of a detailed genomic map. Bacterial artificial chromosome (BAC) library construction is underway to generate contigs for scaffolding sequencing information and for linking to the AFLP map. Due to the simple inheritance of aggressiveness that was genetically caused by only a few loci in the Z-3639 x R-5470 cross, there is a good chance to truly dissect quantitative variation at the molecular

level. These advances will unravel knowledge gaps in the genetics of *G. zeae* and offer exciting possibilities for deployment of effective FHB management.

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8 Summary

Fusarium head blight (FHB), caused by *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe), is one of the principal diseases responsible for extensive damage in wheat fields and contamination of grain with the mycotoxins deoxynivalenol (DON) and nivalenol (NIV), rendering the harvest unsafe for human and animal consumption. Control of FHB is difficult because of the complex nature of host-pathogen-environment interaction and the nonavailability of highly effective fungicides. Agronomic practices and resistance breeding, therefore, offer the best strategies for disease management. Mapping by molecular markers provides an accurate approach for genetic analyses of simple and complex traits particularly pathogenicity, aggressiveness, and mycotoxin production. Pathogenicity, as defined here, is the ability to cause disease whereas aggressiveness is the quantity of disease induced by a pathogenic isolate on a susceptible host in which isolates do not interact differentially with host cultivars. The project aims to (1) map pathogenicity and aggressiveness of *G. zeae* based on a published genetic map (2) estimate genetic diversity of four parent isolates by PCR-based markers (3) examine the inheritance of pathogenicity, aggressiveness, mycotoxin type (DON/NIV), and DON production on a phenotypic basis, (4) analyse genetic covariation among aggressiveness, DON, and fungal colonization, (5) and compare aggressiveness of 42 isolates in greenhouse and field environments.

Two crosses of *G. zeae* using *nit* (nitrate nonutilizing) marker technique were performed: (1) pathogenic DON-producing Z-3639 (Kansas, USA) x nonpathogenic NIV-producing R-5470 (Japan) belonging to lineage 7 and 6, respectively, and (2) DON-producing FG24 (Hungary) x FG3211 (Germany), both aggressive lineage 7 isolates. For the first cross, 99 progeny segregated in a consistent 61:38 for pathogenicity: nonpathogenicity in a two-year greenhouse experiment. Among the 61 pathogenic progeny, disease severity, measured as percentage infected spikelets, varied significantly (P = 0.01). Heritability for aggressiveness was high. Pathogenicity locus was mapped on linkage group IV near loci *PIG1* (red pigment production), *TOX1* (trichothecene toxin amount), and *PER1* (perithecial production) explaining 60%, 43%, and 51% of the phenotypic variation, respectively. Two large aggressiveness QTLs were mapped on linkage group I linked to the locus *TRI5* (trichodiene synthase in the trichothecene gene cluster) and an amplified fragment length polymorphism (AFLP) marker (*EAAMTG0655K*), explaining 51% and 29% of the observed phenotypic variation, respectively. These unlinked loci suggest that genetic basis between pathogenicity and aggressiveness were different. *TRI5* is located in the same gene cluster as a previously

identified gene known as *TRI13,* which determines whether DON or NIV will be produced. DON-producing progeny were, on average, twice as aggressive as were those producing NIV. Loci were only detected in the two linkage groups mentioned from the nine linkage groups present in the map.

For the second cross FG24 x FG3211 with 153 progeny, head blight rating and relative plot yield were used as aggressiveness traits. DON production was measured by a commercial kit enzyme immunoassay. These three traits were quantitatively inherited among 153 progeny across three environments. Repeatabilities within each environment were medium to high but heritabilities across environments were medium only due to high progeny-environment interaction. DON was a less environmentally stable trait than aggressiveness. Transgressive segregants were detected frequently. This implies that even a cross within a lineage could lead to an increase in aggressiveness. Mapping of this cross was not initiated because the parents were not polymorphic enough to construct a genetic map. Instead, the parents were analysed for polymorphism in comparison to the parents of the first cross using 31 AFLP primer combinations and 56 random amplified polymorphic DNA (RAPD) primers. Polymorphism between Z-3639 and R-5470 was about three to four times higher than between FG24 and FG3211. Cluster analysis revealed that R-5470 was genetically separated from the other three parents, thus confirming the lineage assignments.

Among preselected 50 progeny from the same field experiments that showed normal distribution for aggressiveness - head blight rating, fungal colonization, and DON production were correlated ($r = 0.7$, $P = 0.01$). Fungal colonization measured as *Fusarium* exoantigen (ExAg) content using enzyme-linked immunosorbent assay (ELISA) varied also quantitatively, but heritability was lower due to high progeny-environment interaction and error. Strong correlations among all traits indicate control by similar genes or gene complexes. No significant variation was observed for DON/ExAg ratio. Aggressiveness traits and DON production were more environmentally stable compared to *Fusarium* ExAg content. Our findings imply that aggressiveness may have other components apart from mycotoxin production. Genotypic variation for aggressiveness among the 42 progeny in one greenhouse and three field environments was significant and their correlation was moderate ($r = 0.7$, $P = 0.01$). High heritability in both environments again indicates that aggressiveness was a relatively stable trait, although methods of inoculation differed, i.e., injection for greenhouse and spraying for field experiments. Greenhouse aggressiveness could predict aggressiveness in the field, and thereby should reduce costs for resistance and phytopathological studies.

In conclusion, we consider *G. zeae* as medium-risk pathogen with the potential to evolve to a higher level of aggressiveness due to sexual recombination. Erosion of quantitative resistance in FHB cannot be ignored, especially if host resistances with oligogenic inheritance, e.g. Sumai 3 from China, are used on a large acreage. Consequently, the rather simple inheritance of pathogenicity and aggressiveness in *G. zeae* could lead to a gradual increase of aggressiveness. These results should enhance efforts of plant breeders to use several, genetic distinct sources of resistance in order to avoid possible FHB outbreaks in the future.

9 Zusammenfassung

Ährenfusariosen, die im Wesentlichen von *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) verursacht werden, können zu hohen Ertragsverlusten und der Kontamination mit Mykotoxinen führen. Hauptsächlich werden die Mykotoxine Deoxynivalenol (DON) und Nivalenol (NIV) produziert, die eine gesundheitsschädigende Wirkung auf Mensch und Tier haben. Wegen der hohen Wirt-Pathogen-Umwelt-Interaktion sowie wenig effizienter Fungizide ist die Bekämpfung von Ährenfusariosen problematisch. Pflanzenbauliche Maßnahmen und Resistenzzüchtung können zu einer Verminderung des Pilzbefalls führen. Die Kartierung mit molekularen Markern bietet die Möglichkeit, sowohl einfach wie auch komplex vererbte Merkmale zu untersuchen. Pathogenität und Aggressivität stellen zwei wichtige Bedingungen der Infektion und der Befallsentwicklung von Ährenfusariosen dar. Pathogenität wird dabei als die Fähigkeit eines Isolats definiert, Krankheitssymptome auf einem anfälligen Wirt hervorzurufen. Aggressivität beschreibt die Stärke des Befalls, wenn die Isolate nicht differentiell mit den Wirtsgenotypen interagieren. Zielsetzung des Projektes war (1) die Kartierung der Pathogenität und der Aggressivität von *G. zeae*, aufbauend auf einer bereits publizierten genetischen Karte, (2) die Analyse der Diversität aller Eltern-Isolate mit PCR-basierenden Markern, (3) die Untersuchung der Vererbung von Pathogenität, Aggressivität, Mykotoxin-Typ (DON/NIV) und DON-Produktion, (4) die Analyse der genetischen Kovariation zwischen Aggressivität, DON und Myzelwachstum und (5) der Vergleich der Aggressivität von 42 Isolaten im Gewächshaus und Feld.

Es wurden zwei Kreuzungen mittels *nit* (*nitrate nonutilizing*)-Markern durchgeführt, zum einen wurde das pathogene, DON-produzierende Isolat Z-3639 aus Kansas (USA) mit dem nicht-pathogenen, NIV- produzierenden Isolat R-5470 (Japan) der Abstammungslinien 7 bzw. 6 gekreuzt, zum anderen wurden die beiden DON-produzierenden, aggressiven Isolate FG24 (Ungarn) und FG3211 (Deutschland) der Abstammungslinie 7 gekreuzt. Die 99 Nachkommen der ersten Kreuzung spalteten in einem zweijährigen Gewächshausversuch qualitativ in einem Verhältnis 61:38 für das Merkmal Pathogenität. Die Aggressivität, gemessen als Prozent infizierter Ährchen, variierte signifikant (P=0,01) zwischen den 61 pathogenen Nachkommen. Die Heritabilität für die Aggressivität war in dieser Kreuzung hoch. Die Pathogenität wurde auf der Kopplungsgruppe IV nahe den Loci *PIG1* (Pigmentierung), *TOX1* (Trichothecen-Gehalt), und *PER1* (Perithezienproduktion) lokalisiert. Sie erklärten 60%, 43% bzw. 51% der phänotypischen Varianz. Zwei Loci für die

Aggressivität waren auf der Kopplungsgruppe I mit *TRI5* (Trichodiensynthase) und einem AFLP- (*amplified fragment length polymorphism*) Marker (*EAAMTG0655K*) gekoppelt und erklärten 51% bzw. 29% der phänotypischen Varianz. *TRI5* befindet sich im gleichen Gen-Cluster wie *TRI13,* das über die Bildung von DON oder NIV entscheidet. Die DON-produzierende Teilpopulation war im Durchschnitt zweimal so aggressiv wie die NIV-produzierende Teilpopulation. Es wurden keine weiteren Loci für die Pathogenität oder Aggressivität auf den restlichen sieben Kopplungsgruppen dieser Karte gefunden. Daraus lässt sich schlussfolgern, dass beide Merkmale in dieser Kreuzung auf unterschiedliche Weise vererbt werden.

Für die zweite Kreuzung FG24 x FG3211 mit 153 Nachkommen wurden die Ährenbonitur und das relative Ährengewicht als Merkmal für die Aggressivität in einem Feldversuch über drei Umwelten ermittelt. Zusätzlich wurde die DON-Produktion mittels eines handelsüblichen Immunotestes gemessen. Alle drei Merkmale zeigten eine quantitative Verteilung. Trotz einer mäßigen bis hohen Wiederholbarkeit in den Einzelumwelten, war die Heritabilität in der Serienverrechnung wegen der hohen Genotyp-Umwelt-Interaktion nur mäßig. Die DON-Produktion zeigte eine geringere Umweltstabilität als die Aggressivität. Transgressionen wurden häufig beobachtet. Dies zeigt, dass auch eine Kreuzung innerhalb einer Abstammungslinie zu einer höheren Aggressivität führen kann. Die Kartierung der zweiten Kreuzungsnachkommenschaft konnte nicht durchgeführt werden, weil der Polymorphismus zwischen den Eltern zu gering war. Statt dessen wurden die Eltern dieser Kreuzung mittels 31 AFLP-Primerkombinationen und 56 RAPD- (*random amplified polymorphic DNA*)-Primern mit den beiden Eltern der ersten Kreuzung verglichen. Der Polymorphiegrad zwischen Z-3639 und R-5470 war etwa drei bis viermal höher als zwischen FG24 und FG3211. Die Cluster-Analyse ergab, dass R-5470 von den anderen drei Eltern genetisch stark verschieden war, wodurch die Zugehörigkeit zu einer anderen Abstammungslinie als die der restlichen Isolate gerechtfertigt werden kann.

Bei fünfzig selektierten Nachkommen, die im Feldversuch über drei Umwelten eine Normalverteilung für die Aggressivität zeigten, korrelierten die Ährenbonitur, das Myzelwachstum und die DON Produktion relativ gut ($r = 0,7$; $P = 0,01$). Auch das Myzelwachstum, gemessen als *Fusarium*-Exoantigen (ExAg) mittels ELISA (*enzyme-linked immunosorbent assay*), variierte quantitativ. Die Heritabilität für dieses Merkmal war jedoch aufgrund der hohen Bedeutung von Genotyp-Umwelt-Interaktion und Fehler niedrig. Die Korrelation zwischen allen Merkmalen zeigt, dass sie von ähnlichen Genen oder Genkomplexen kontrolliert werden. Es wurde keine signifikante Variation für das

DON/ExAg-Verhältnis nachgewiesen. Die Aggressivitätsmerkmale und die DON-Produktion waren umweltstabiler als der *Fusarium*-ExAg-Gehalt. Diese Ergebnisse belegen, dass neben der Mykotoxinproduktion noch andere Merkmale zur Aggressivität beitragen.

Die genotypische Variation für die Aggressivität bei 42 Nachkommen im Gewächshaus und an drei Feldumwelten war signifikant (P = 0,01), die Korrelation war mäßig hoch (r = 0,7; P = 0,01). Dies zeigt, dass die Aggressivität stabil vererbt wurde, obwohl zwei unterschiedliche Inokulationsmethoden angewandt worden waren. Die Einzelährcheninjektion im Gewächshaus einerseits und die Sprühinfektion im Feld andererseits. Daher können die Ergebnisse aus den Gewächshausversuchen zur Vorhersage der Aggressivität im Feld genutzt und damit die Kosten für solche Untersuchungen gesenkt werden.

Zusammenfassend sollte *G. zeae* als Pathogen mit einem mittleren Risiko zur Überwindung von Resistenzen eingestuft werden. Allerdings kann evolutionär durchaus ein höheres Aggressivitätsniveau durch sexuelle Rekombination erreicht werden. Die Erosion quantitativer Resistenz bei Ährenfusariosen sollte dabei nicht vernachlässigt werden. Dies gilt besonders dann, wenn Wirtsgenotypen mit oligogenischer Vererbung der Resistenz, wie beispielsweise die chinesische Sorte ‘Sumai 3’, einen großen Anteil der Anbaufläche haben. Die einfache Vererbung der Pathogenität und Aggressivität bei *G. zeae* kann dann zu einer graduellen Erhöhung der Aggressivität führen. Daher sollten Pflanzenzüchter mehrere, genetisch unterschiedliche Resistenzquellen für die Sortenentwicklung nutzen, um in Zukunft größer Epidemien durch Ährenfusariosen zu vermeiden.

ACKNOWLEDGEMENTS

I express my sincere thanks and gratitude to the following people:

To Priv. Doz. Dr. Thomas Miedaner for supervising the research project. His advices and encouragement led to a successful completion of my Ph.D. degree. To Bianca Schneider for her expertise in field and greenhouse experiments. To Prof. Dr. Hartwig H. Geiger for supporting me in the annual extension of my scholarship and recommending me to the Eiselen Foundation travel funds.

To our Kansas State collaborators, Dr. Robert L. Bowden, for his great insights regarding the project and warm hospitality during my visit to the 2003 American Phytopathological Conference in Charlotte, North Carolina, Dr. James E. Jurgenson for his mapping analyses, and Dr. John F. Leslie for his comments and suggestions on our first paper.

To Dr. Heiko Parzies for his enthusiasm in the AFLP technique and assistance in population genetic analyses.

To Dr. Frank Rabenstein and Monika Wesermann for their exoantigen analyses of some progeny of our *G. zeae* population.

To Prof. Dr. Heinrich Buchenauer, Prof. Dr. Rolf Blaich, and Prof. Dr. Anne Valle Zárate for serving as members of the examination committee.

To the members of the Rye Working Group - Lucija, Olaf, Bärbel, Kerstin, Heike, Mark, and Gabi - for helping me in many ways and to all the "Hiwis" who helped in spore counting, disease rating, harvesting and processing samples for mycotoxin analyses.

To Manfred Buck-Finkele and Sabine Pöschel for their field assistance in Oberer Lindenhof.

To the greenhouse technicians - Norbert Köster, Detlef Stabenow, and Diego Ramos-Carrion - for taking care of my potted plants.

To my Ph.D. student colleagues, Katinka, Jasmina, Zoran, Adel, Zeljko, and Andres for their help in computer programs, statistics, plant breeding techniques, etc.

To Andrea, Dorothee, and Dr. Evelyn Möller for their assistance on molecular aspects of the project.

To Dr. Chris Schön and Gerlinde Hartmann for assisting in all my official transactions in Hohenheim.

To my dear wife Casey, my "German kids" Jojo and Chelsea for being my source of joy and inspiration, for making me feel at home in Germany.

The research was supported by a Ph.D. grant from the German Academic Exchange Service (DAAD), Bonn.

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