Sexual Recombination in *Gibberella zeae*

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ABSTRACT

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We developed a method for inducing sexual outcrosses in the homothallic Ascomycete fungus *Gibberella zeae* (anamorph: *Fusarium graminearum*). Strains were marked with different nitrate nonutilizing (*nit*) mutations, and vegetative compatibility groups served as additional markers in some crosses. Strains with complementary *nit* mutations were cocultured on carrot agar plates. Ascospores from individual perithecia were plated on a minimal medium (MM) containing nitrate as the sole nitrogen source. Crosses between different *nit* mutants segregated in expected ratios (3:1 nit^-:nit^+) from heterozygous perithecia. Analysis of vegetative compatibility groups of progeny of two crosses indicated two and three vegetative incompatibility (*vic*) genes segregating, respectively. For rapid testing of sexual recombination between *nit* mutants, perithecia were inverted over MM to deposit actively discharged ascospores. Development of prototrophic wild-type colonies was taken as evidence of sexual recombination. Strains of *G. zeae* group 2 from Japan, Nepal, and South Africa, and from Indiana, Kansas, and Ohio in the United States were sexually interfertile. Four group 1 strains were not interfertile among themselves or with seven group 2 strains. Attempts to cross *G. zeae* with representatives of *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. crookwellense*, *F. oxysporum*, and three mating populations of *G. fujikuroi* were not successful.

Additional keywords: dryland foot rot, ear rot, Fusarium head blight, maize, scab, stalk rot, wheat.

The Ascomycete fungus *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) causes crown rot and head scab of wheat and barley, stalk rot and ear rot of maize, and crown rot of carnation (30,43,44). Scab, also called Fusarium head blight, has recently re-emerged as a devastating disease of wheat and barley throughout the world (28). In addition to direct yield losses, scab reduces grain quality (42), and harvested grain is often contaminated with the mycotoxins nivalenol, deoxynivalenol (vomitoxin), and zearalenone (27). Although *F. culmorum*, *F. poae*, and *Microdochium nivale* can also cause scab symptoms (33), *G. zeae* is the most important causal agent of wheat scab in the United States (46,48). Details of scab disease epidemiology, losses, and control have been well reviewed (2,3,28,33,43).

G. zeae also is fermented commercially (18) as part of the production process for zearanol, which is sold as a bovine growth stimulant (Ralgro; Pitman-Moore, Terre Haute, IN). In addition, a strain identified as *F. graminearum* is grown as a mycoprotein food supplement for human consumption called Quorn (Marlow Foods Ltd., Stokesley, Cleveland, England) (47). However, the identification of the Quorn mycoprotein fungus was recently questioned (31).

Some progress has been made in discerning the population structure of *G. zeae*. Australian researchers divided the species into two distinct ecological groups (8,9,17,38). Group 1 is primarily soilborne and causes crown rot or dryland foot rot of wheat, barley, and other grasses. It is found in arid regions of Australia, South Africa, and North America. Perithecia of group 1 were found in the field on a few occasions (17). Perithecia have never been obtained from monoconidial cultures of group 1 and only rarely in paired cultures (17). Members of group 1 are presumed to be heterothallic, infertile, or both (17). Group 2 is primarily airborne and causes scab of small grains, stalk or ear rot of maize, and carnation crown rot. Members of this group are homothallic and often produce abundant perithecia in the field and under laboratory conditions on some media (5,17,43,45).

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Cullen et al. (13) distinguished two types within group 2 of *G. zeae*. Type A is pathogenic to maize, grows rapidly, forms red-pigmented colonies with abundant aerial mycelium, and usually produces low amounts of zearalenone. Type B is nonpathogenic to maize, grows slowly, forms appressed brownish-yellow colonies, and usually produces high levels of zearalenone. Type A was most prevalent, comprising 95% of the strains. The relationship between types A and B is unknown.

Since *G. zeae* group 2 is homothallic (15), it has been suggested that perithecia served only for survival and to produce genetically uniform inoculum (11). On the other hand, Bowden and Leslie (5) suggested that the high genotypic diversity of *G. zeae* could be due to occasional outcrossing. *Aspergillus nidulans* is an example of a homothallic Ascomycete that is capable of outcrossing (35). All of the asci in an ascocarp normally arise from a single fertilization event (16). The diploid portion of the life cycle is restricted to meiosis and is entirely contained within the ascocarp. Individual ascocarps are either heterozygous (outcrossed) or homozygous (selfed) (16).

If *G. zeae* were capable of sexual recombination, it might allow natural populations to adapt more quickly to selective pressures such as cultivar resistance or fungicides. The ability to manipulate sexual recombination in the laboratory would facilitate study of inheritance, gene mapping, and genetic exchange between populations. Finally, the ability to select progeny from crosses between strains with desirable characters (e.g., higher yield and better growth traits) is an attractive alternative to traditional industrial strain improvement protocols that rely primarily on multiple rounds of mutation and selection for strain improvement.

The objectives of this study were to (i) determine if sexual outcrossing occurs in *G. zeae*, (ii) develop routine crossing methods, and (iii) investigate potential barriers to sexual fertility between different strains of *G. zeae* or its relatives. A preliminary report of this work has been published (7).

MATERIALS AND METHODS

Fungal cultures. Strain numbers, geographic origin, host, and source are listed in Table 1. Kansas State University strain num-

bers are used throughout the text. All cultures were purified by single-spore isolations with a micromanipulator. Identifications were provided by the source of each strain and were confirmed using standard methods (30). Strains were maintained for short periods on slants of a complete medium described by Correll et al. (12). Cultures were stored for longer periods as suspensions of hyphal fragments and conidia in 15% glycerol at –80°C.

Genetic markers. Each parent in all crosses was marked with a different class of nitrate nonutilizing (*nit*) mutation. Four classes (*nit1*, *nit3*, NitM, and *nnu*) of *nit* mutants were previously reported in *G. zeae* (5,21,22). The *nit* mutants were obtained as fast-growing sectors on minimal medium (MM) amended with 1.5 or 2.5% chlorate and 0.16% L-asparagine (12). The *nit* phenotypes were determined on basal medium amended with different nitrogen sources (12). The mutant designations were appended to the original strain number. Thus, Z-3634 *nit1* and Z-3634 *nit3* are *nit1* and *nit3* mutants, respectively, of strain Z-3634.

Vegetative compatibility groups (VCGs) served as additional markers in some crosses. Since VCG is controlled by alleles at multiple vegetative incompatibility (*vic*) loci (23), some progeny should be in recombinant VCGs (i.e., VCGs that are different from those of either parent). Methods for pair-wise testing of VCGs using *nit* mutants of *G. zeae* were described by Bowden and Leslie (5).

Mycelial plug crossing method. All crosses were done in 60-mm plastic petri plates containing carrot agar (19). Plates were incubated at 24°C under a mixture of fluorescent cool white and black lights (Sylvania 350BL; GTE Corp., Stamford, CT) with a 12-h photoperiod. Plates were arranged right side up in a single layer on the incubator shelves.

Small (1 mm^3) mycelial plugs from cultures of each parent strain were placed on opposite sides of the petri plate. On day 7, approximately 1 ml of sterile 2.5% (vol/vol) Tween 60 solution was added to each plate. Aerial mycelia were knocked down with

TABLE 1. *Fusarium* and *Gibberella* strains used in this study

Species	KSU no.[x]	Other no.	Donor	Geographic origin	Host or substrate	Comment
F. acuminatum	5019	R-5701	P. E. Nelson	Georgia, USA	*Claviceps paspali* honeydew	
F. acuminatum	5020	R-6666	P. E. Nelson	Delaware, USA	Ragweed stem	
F. avenaceum	5017	R-6550	P. E. Nelson	California, USA	Carnation	
F. avenaceum	5018	R-6763	P. E. Nelson	Pennsylvania, USA	Poultry feed	
F. crookwellense	6574	R-4812	P. E. Nelson	Australia	Pine	
F. crookwellense	6580	R-6778	P. E. Nelson	Columbia	Carnation	
F. crookwellense	6585	R-9474	P. E. Nelson	Venezuela	Carrot	
F. crookwellense	4833	MRC2878	W. F. O. Marasas	Michigan, USA	Soil	
F. crookwellense	4834	MRC4643	W. F. O. Marasas	South Africa	Potato	
F. crookwellense	4835	MRC4682	W. F. O. Marasas	South Africa	Potato	
F. culmorum	6575	R-5452	P. E. Nelson	Australia	Maize	
F. culmorum	6576	R-5626	P. E. Nelson	Wisconsin, USA	Red clover	
F. culmorum	6577	R-5906	P. E. Nelson	Oregon, USA	Sugar pine	
F. culmorum	6581	R-7505	P. E. Nelson	Idaho, USA	Lentil	
F. culmorum	6582	R-8504	P. E. Nelson	Denmark	Barley	
F. culmorum	6583	R-8515	P. E. Nelson	Oregon, USA	Garlic	
F. oxysporum f. sp. *apii*	2520	O-1	J. E. Puhalla	California, USA	Celery	
F. oxysporum f. sp. *cubense*	2534	O-1222	J. E. Puhalla	Australia	Banana	
F. oxysporum f. sp. *lycopersici*	2527	O-1078	J. E. Puhalla	Florida, USA	Tomato	
F. oxysporum f. sp. *medicaginis*	2522	O-6	J. E. Puhalla	California, USA	Alfalfa	
F. oxysporum f. sp. *radicis-lycopersici*	2530	O-1090	J. E. Puhalla	Ontario, Canada	Tomato	
F. oxysporum f. sp. *tracheiphilum*	2847	923 *nitA*	J. E. Puhalla	Mississippi, USA	Cowpea	*nit1* mutant
G. fujikuroi	C-1775	MRC-2290	W. F. O. Marasas	Unknown	Rice	Mating type C–
G. fujikuroi	C-1993	M 1148	P. E. Nelson	Taiwan	Rice	Mating type C+
G. fujikuroi	B-3852	ATCC 201264	…[y]	Progeny of cross	Progeny of cross	Mating type B+
G. fujikuroi	B-3853	ATCC 201265	…	Progeny of cross	Progeny of cross	Mating type B–
G. fujikuroi	A-853	M 1140 and A408 *nit1*	P. E. Nelson	California, USA	Maize	Mating type A–
G. zeae group 1	4820	R-4992	P. E. Nelson	Australia	Wheat	
G. zeae group 1	4822	R-5291	P. E. Nelson	Australia	Barley	
G. zeae group 1	4824	R-6562	P. E. Nelson	Washington, USA	Wheat	
G. zeae group 1	5027		…	Kansas, USA	Wheat	
G. zeae group 2	Z-6587	Crawford 5	E. B. Smalley	Ohio, USA	Maize	Type B[z]
G. zeae group 2	Z-6586	Wood 1	E. B. Smalley	Ohio, USA	Maize	Type B[z]
G. zeae group 2	Z-5048	R-5469	P. E. Nelson	Japan	Barley	Produces nivalenol, fusarenon-X
G. zeae group 2	Z-6296	R-9434	A. E. Desjardins	Kashi, Nepal	Maize	
G. zeae group 2	Z-6311	Nep 21	A. E. Desjardins	Lamjung, Nepal	Maize	
G. zeae group 2	Z-6317	Nep 44	A. E. Desjardins	Lamjung, Nepal	Maize	
G. zeae group 2	Z-3634		…	Kansas, USA	Wheat	
G. zeae group 2	Z-3635		…	Kansas, USA	Wheat	
G. zeae group 2	Z-3636		…	Kansas, USA	Wheat	
G. zeae group 2	Z-3639		…	Kansas, USA	Wheat	
G. zeae group 2	Z-3651		…	Kansas, USA	Wheat	
G. zeae group 2	Z-3654		…	Kansas, USA	Wheat	
G. zeae group 2	Z-4218	ATCC 48067	…	Indiana, USA	Maize	Type B, *nnu* mutant[z]
G. zeae group 2	Z-4838	MRC 1115	W. F. O. Marasas	South Africa	Maize	
G. zeae group 2	Z-4839	MRC 5049	W. F. O. Marasas	South Africa	Wheat	
G. zeae group 2	Z-4840	MRC 5059	W. F. O. Marasas	South Africa	Wheat	
G. zeae group 2	Z-4867	MRC 5061	W. F. O. Marasas	South Africa	Wheat	

[x] KSU = Kansas State University.
[y] … = Our own strain.
[z] All *G. zeae* group 2 strains are type A sensu Cullen et al. (13) unless noted otherwise.

a sterile bent glass rod while plates were rotated several times to spread the solution. Plates were returned to the incubator, and abundant perithecia usually covered the plate by day 14.

On days 17 to 18, ascospore cirrhi on individual perithecia were sampled along the interface line between the parental colonies. Cirrhi were carefully removed under a dissecting microscope with a sterile needle. Each cirrhus, approximately 1,000 to 5,000 ascospores, was placed in a tube containing 4.5 ml of sterile 2.5% Tween 60 solution. The tube was mixed for 5 to 10 s with a Vortex mixer (Scientific Industries, Inc., Bohemia, NY), after which 300 µl was spread on 100-mm plates of MMTS medium, in which MM is amended with 0.05% (vol/vol) tergitol type NP-10 (37) and 2% (wt/vol) L-sorbose instead of 3% sucrose. MMTS restricts colony radial growth, facilitating colony counts.

After 5 to 7 days of incubation, *nit* mutants produced thin, wispy, brownish colonies with little or no aerial mycelium. In contrast, prototrophic wild-type strains produced dense button-like colonies with cottony pink or white aerial mycelium. A few wild-type strains produced dense colonies covered with orange sporodochia and little aerial mycelium on MMTS. Prototrophic wild types were assumed to be the result of sexual recombination and not revertants to wild type. Unpaired carrot agar cultures of marked parental strains served as negative controls for each cross.

Segregation of markers. We used the mycelial plug method to study segregation of *nit* and VCG markers in two crosses: Z-3651 *nit1* × Z-3654 NitM and Z-3634 NitM × Z-3635 *nit3*. These four strains were previously found to be in different VCGs (5). Plates from putative heterozygous perithecia with a well-spaced mixture of wild-type and *nit* mutant colonies were selected. The numbers of wild-type and *nit* mutant colonies were counted. Small plugs (1 mm^3) from the margins of individual *nit* mutant colonies were transferred to complete medium and then tested for *nit* phenotype. Some progeny were paired with parent strains or other progeny to determine VCGs. Selected progeny in recombinant VCGs were backcrossed to parents to confirm the number of segregating *vic* loci.

Crossing method evaluation. The mycelial plug method was compared with two other crossing methods. In the mixed-inoculum method, 0.1 ml of a spore suspension containing approximately 1×10^4 conidia per ml of each parental strain was spread on carrot agar plates with a sterile bent glass rod. This resulted in a dense mixed lawn of parental colonies. Subsequently, plates were treated the same as in the plug method, except cirrhi were sampled randomly from the plates.

In the spermatization method, a mycelial plug of one parent strain was placed in the center of a carrot agar plate. On day 7, 2 ml of a conidial suspension containing 10^5 to 10^6 conidia per ml of the other parent strain was added to the culture. Aerial mycelium was knocked down with a sterile bent glass rod while plates were rotated several times to spread the suspension. This protocol is similar to that used to spermatize female cultures of *G. fujikuroi* to initiate crossing (19). Cirrhi were sampled randomly from the plates.

Perithecia that yielded approximately 25% wild-type and 75% *nit* mutant colonies were considered to be heterozygous. Perithecia that yielded only *nit* mutant progeny were considered homozygous. MMTS plates with fewer than 20 colonies were discarded to control the error rate at $\alpha < 0.01$ for misclassifying heterozygous perithecia as homozygous. Some cirrhi contained a low percentage of wild types, which were presumably contaminants from airborne ascospores within the petri plate. A χ^2_1 test for goodness-of-fit for a 3:1 *nit*:wild type ratio was conducted in these cases. Perithecia with less than 25% wild type and $P < 0.01$ for 3:1 segregation were considered to be homozygous.

The proportion of heterozygous perithecia was determined for each petri plate. The proportion was given an arcsine square root transformation for analysis and back-transformed for presentation. The experiment was analyzed as a completely randomized design with three treatments. Means were separated using Fisher's protected least significant difference with $\alpha = 0.05$. The experiment was performed three times with different sets of mutants: (i) Z-3634 NitM × Z-3635 *nit3*; (ii) Z-3634 *nit3* × Z-3636 NitM; and (iii) Z-3634 NitM × Z-3636 *nit1*. For the first two runs, there were three replicate plates and 10 perithecia were sampled per plate. For the third run, there were five replicates and six perithecia were sampled per plate.

Interfertility assay. The interfertility of a set of *G. zeae* group 2 and group 1 strains was tested. Attempts were also made to cross *G. zeae* with representatives of several species of *Gibberella* or *Fusarium.* Plates were inoculated by the mycelial plug method. An ascospore print assay method was developed for more efficient detection of heterozygotes. Carrot agar plates with mature perithecia were inverted over 60-mm MMTS plates for 8 to 16 h. Actively discharged ascospores formed a visible spore print on the target plate. The ascospore print for an individual perithecium was approximately 2 mm in diameter. Interfertile pairings were detected by the presence of wild-type recombinant colonies after 5 to 7 days of incubation. Interfertility experiments were repeated once.

RESULTS

Z-3651 *nit1* × Z-3654 NitM. Progeny were collected from two putative heterozygous perithecia. There were 77 *nit* mutants and 30 wild-type recombinants. The ratio was not significantly different ($\chi^2_1 = 0.53$, $P = 0.47$) from the expected 3:1 segregation ratio for a cross between two haploid auxotrophic mutants. A total of 67 *nit* mutants were identified to phenotype. The NitM-*nit1* double-mutant phenotype could not be distinguished from the NitM single-mutant phenotype. A total of 42 progeny appeared to be NitM and 25 were *nit1*, a ratio that was not significantly different ($\chi^2_1 = 0.48$, $P = 0.49$) from the expected 2:1 ratio.

Progeny that were single NitM mutants rather than NitM-*nit1* double mutants were identified by their ability to complement a *nit1* mutant in a preliminary VCG experiment. Four of these NitM progeny were used along with NitM mutants from each parent to test the VCG of 25 *nit1* progeny. In addition to both parental VCGs, two recombinant VCGs were found among the *nit1* progeny. All four VCGs also were represented among the four NitM prog-

TABLE 2. Results of vegetative compatibility group (VCG) tests of progeny from Z-3634 NitM × Z-3635 *nit3* and backcrosses of progeny to parents

VCGs of parents[x]	No. progeny tested	VCGs of progeny	*vic* loci[y]
P1 × P2[z]	48	P1, P2, R1, R2, R3, R4, R5, R6	3
P1 × R1	6	P1, R1, R3	2
P1 × R2	7	P1, R2	1
P1 × R3	5	P1, R3	1
P1 × R4	7	R2, R4, R6	2
P1 × R5	6	P1, R2, R5	2
P1 × R6	8	P1, R6	1
P2 × R1	5	P2, R1	1

[x] P1 and P2 are arbitrary designations for VCGs of strains Z-3634 NitM and Z-3635 *nit3*, respectively. R1 through R6 are arbitrary VCG designations of recombinant progeny.
[y] Minimum number of *vic* loci segregating.
[z] Original cross.

TABLE 3. Effect of different crossing methods on proportion of heterozygous perithecia

	Experiment[z]		
Crossing method	1	2	3
Mycelial plug	0.354 a	0.200 a	0.216 a
Mixed inoculum	0.236 a	0.067 ab	0.102 a
Spermatization	0.011 b	0.000 b	0.031 a

[z] Back-transformed means of proportion of heterozygous perithecia. Means within a column followed by the same letter are not significantly different according to Fisher's protected least significant difference ($\alpha = 0.05$).

eny. Among the *nit1* progeny, the ratio of the four VCGs was 9:7:5:4, which was not significantly different from a 1:1:1:1 ratio ($\chi^2_3 = 2.36$, $P = 0.50$).

The presence of only four VCGs among the progeny indicated that the two parental strains differed at only two *vic* loci. By inference, the two recombinant VCGs also differed at only two *vic* loci, but each differed from the parental types at one *vic* locus. Occasional weak complementation was observed in vegetative pairings of parents and recombinants, but not in pairings of parents or pairings of recombinants. Thus, differences at two *vic* loci were required for complete vegetative incompatibility among the progeny of this cross.

Z-3634 NitM × Z-3635 *nit3*. Progeny were collected from 11 putative heterozygous perithecia. There were 207 *nit* mutants and 80 wild-type recombinants, which was not significantly different ($\chi^2_1 = 1.26$, $P = 0.26$) from the expected 3:1 ratio. We identified the phenotype of 124 *nit* mutants. In this cross, each progeny phenotypic class could be distinguished including the recombinant NitM-*nit3* double mutant. The NitM:*nit3*:NitM-*nit3* ratio was 43:44:37 and was not significantly different ($\chi^2_2 = 0.69$, $P = 0.88$) from the expected 1:1:1 ratio.

Six randomly chosen NitM progeny were used along with NitM mutants of each parent to test the VCG of 42 *nit3* progeny. Both parental VCGs (arbitrarily designated P1 and P2) and three recombinant VCGs (R1, R2, and R3) were clearly identified among the *nit3* progeny. Three additional recombinant VCGs (R4, R5, and R6) were indicated by patterns of weak interactions with the NitM strains. One *nit3* tester strain was selected to represent each of the eight putative VCGs. These eight *nit3* testers were then used to select eight complementary NitM testers from the progeny of the cross.

Results were consistent with the segregation of three *vic* loci. For confirmation, one *nit3* progeny from each of the six recombinant VCGs was backcrossed to NitM mutants of one or both parents. Five to eight *nit3* or NitM progeny from each backcross were paired with the VCG tester strains. One or two *vic* loci appeared to segregate in each backcross (Table 2). A total of eight VCGs were found among all progeny of the backcrosses, thus confirming that three *vic* loci were segregating in the original cross. Weak incompatibility reactions were not consistent, so the gene or genes responsible for the weak interactions could not be identified. The results of these tests demonstrate that classical genetic crosses can be made and routinely analyzed in *G. zeae*.

Crossing method evaluation. The proportion of heterozygous perithecia varied considerably between replicates (data not shown). The mycelial plug method consistently produced the most heterozygotes, and it was significantly better than the spermatization method in two of the three experiments (Table 3). The mixed-inoculum method was consistently intermediate and was significantly better than the spermatization method in one experiment.

Interfertility studies. The ascospore print assay method was used to determine if barriers to sexual fertility existed between different strains of *G. zeae* and representatives of other species of *Gibberella* or *Fusarium*. Some pairings resulted in no fertile perithecia at all. In pairings that produced only homozygous perithecia, a lawn of wispy *nit* mutant colonies was produced. In interfertile pairings, wild-type colonies 2 mm in diameter developed on a lawn of *nit* mutant colonies after 5 to 7 days. The number of wild-type colonies per plate varied from a few to dozens and presumably reflected the number of heterozygous perithecia, since adjacent recombinant wild-type progeny from one heterozygous perithecium quickly coalesced into a single colony. The pattern of wild-type colonies on the MMTS target plate also reflected the pattern of heterozygous perithecia on the carrot agar plate. When only one parent had good female fertility, a semicircular pattern of wild types was produced.

Seven strains of *G. zeae* group 2 from Japan, Kansas, and South Africa were generally interfertile (Table 4). Strain Z-5048 from Japan produced a few perithecial initials, but no mature perithecia in any cultures. However, it was able to cross with all other strains except Z-4838. Colony patterns indicated that Z-5048 was a very good male in most crosses. Similarly, Z-4838 produced only a few mature perithecia in some cultures. Nevertheless, it served as a male with five group 2 strains. In contrast, four strains of *G. zeae* group 1 were not interfertile among themselves or with group 2 strains.

In another experiment, three group 2 strains from Nepal were interfertile with two group 2 strains from Kansas and one from South Africa (Table 5). Within group 2, type A and type B strains were interfertile, but we did not attempt to analyze the genetic basis for the difference in strain type. Pairings of three group 2 strains with two strains of *F. acuminatum*, two of *F. avenaceum*, six of *F. crookwellense*, six of *F. culmorum*, six of *F. oxysporum*, and five of *G. fujikuroi* were not interfertile (Table 5).

DISCUSSION

The homothallic Ascomycete fungus *G. zeae* can outcross and undergo sexual recombination under laboratory conditions. Segregation of *nit* and *vic* alleles was demonstrated in two different crosses between strains of *G. zeae* group 2, and recombinant *nit* phenotypic classes and novel VCGs were obtained. Progeny numbers in different *nit* phenotypic classes were consistent with expected haploid segregation ratios. Similar facultative outcrossing has been described in other homothallic Ascomycetes such as *A. nidulans* (35), *Gaeumannomyces graminis* (34), *Nectria haematococca* (14), and *Sordaria fimicola* (32).

Heterozygous perithecia and sexual recombination in the field have not been found, but several factors suggest that they occur. First, the level of genetic variability in a field population of *G. zeae* with respect to random amplified polymorphic DNAs (RAPDs)

TABLE 4. Interfertility of strains of *Gibberella zeae* group 1 and group 2

Group	Strain[y]	4820	4822	4824	5027	Z-3636	Z-3639	Z-4838	Z-4839	Z-4840	Z-4867	Z-5048	Control
1	4820	N[z]	N	N	N	–	–	N	–	–	–	N	N
1	4822	N	N	N	N	–	–	N	–	–	–	N	N
1	4824	N	N	N	N	–	–	N	–	–	–	N	N
1	5027	N	N	N	N	–	–	–	–	–	–	N	N
2	Z-3636	–	–	–	–	+++	+++	+	+++	+++	+++	++	–
2	Z-3639	–	–	–	–	+++	+++	–	+++	+++	+++	+	–
2	Z-4838	…	N	–	N	+	+	N	++	+	+++	N	N
2	Z-4839	–	–	–	–	+++	+++	+	+++	+++	+++	+++	–
2	Z-4840	–	–	–	–	+++	+++	–	+++	+++	+++	+++	–
2	Z-4867	–	–	–	–	+++	+++	++	+++	+++	+++	+++	–
2	Z-5048	N	N	N	N	+++	++	N	+++	+++	+++	N	N
Control		N	N	N	N	–	–	–	–	–	–	–	N

[y] Parents in columns were *nit1* mutants of indicated strain and parents in rows were *nit3* or NitM mutants.

[z] N = no progeny produced, – = no wild-type colonies, + = one to five recombinant wild-type colonies per amended minimal medium plate, ++ = 6 to 25 wild-type colonies, +++ = >25 wild-type colonies, and … = not done. Each wild-type colony usually represented coalesced colonies of all the progeny from one heterozygous perithecium.

and VCGs is high (5,6,40,41). Second, perithecia of *G. zeae* are common in the field on residue of maize or small grains (17,43). Third, *G. zeae* group 2 outcrosses readily under laboratory conditions. Up to 35% of sampled perithecia were heterozygous when parents were cocultured on carrot agar (Table 3). Although auxotrophic mutants were used, crosses were not forced because *nit* mutants grow well and self freely on carrot agar. Fourth, opportunities for sexual recombination with neighboring colonies may be common. Of 10 wheat heads sampled in a scab epidemic, 9 were colonized by two or more different genotypes of *G. zeae* (6). Fifth, conidia, mycelial fragments, or both may serve as spermatia, which could allow crosses between distant colonies. This situation might be simulated by the spermatization crossing method, which resulted in some heterozygotes. Ascospore prints also often revealed numerous heterozygous perithecia distal to the colony interface in the mycelial plug method. These fertilizations were probably due to movement of conidia, mycelial fragments, or both during the knockdown of aerial mycelium.

TABLE 5. Fertility of crosses of *Gibberella zeae* group 2 tester strains with group 2 strains of diverse origin and representatives of other *Fusarium* or *Gibberella* species

	NitM tester strains			
nit1, *nit3*, or *nnu* strain	Z-3634	Z-3651	Z-4867	Control
G. zeae group 2, type A from Kansas				
Z-3636	+++[z]	+	+	–
Z-3639	++	+	++	–
G. zeae group 2, type A from Nepal				
Z-6296	+++	+++	+++	–
Z-6311	++	++	+++	–
Z-6317	++	+++	+++	–
G. zeae group 2, type B from Indiana and Ohio				
Z-4218	+	+	+	N
Z-6586	+	+	+	–
Z-6587	++	+	+	–
F. acuminatum				
5019	–	–	–	N
5020	–	–	–	N
F. avenaceum				
5017	–	–	–	N
5018	–	–	–	N
F. crookwellense				
4833	–	–	–	N
4834	–	–	–	N
4835	–	–	–	N
6574	–	–	–	N
6580	–	–	–	N
6585	–	–	–	N
F. culmorum				
6575	–	–	–	N
6576	–	–	–	N
6577	–	–	–	N
6581	–	–	–	N
6582	–	–	–	N
6583	–	–	–	N
F. oxysporum				
2520	–	N	–	N
2522	–	–	–	N
2527	–	–	–	N
2530	–	–	N	N
2534	–	–	N	N
2847	–	–	–	N
G. fujikuroi				
853	–	–	–	N
1775	–	–	–	N
1993	–	–	–	N
3852	–	–	–	N
3853	–	–	–	N
Control	–	–	–	

[z] N = no progeny produced, – = no wild-type colonies, + = one to five recombinant wild-type colonies per amended minimal medium plate, ++ = 6 to 25 wild-type colonies, +++ = >25 wild-type colonies. Each wild-type colony usually represented coalesced colonies of all the progeny from one heterozygous perithecium.

If sexual recombination does occur in the field, it could facilitate the assembly of selectively advantageous multilocus genotypes through recombination of favorable alleles from diverse strains. This might improve the ability of *G. zeae* populations to adapt to disease control measures such as resistant varieties, biocontrol organisms, or fungicides. For example, the presence of a sexual cycle in oat crown rust has probably reduced the durability of host resistance genes (20).

Sexual recombination could account for the high diversity of VCGs observed in *G. zeae* (5,6). In the two crosses analyzed in this study, two and six recombinant VCGs were generated, respectively. An advantage of novel VCGs may be to limit the spread of cytoplasmic hypovirulence factors between strains (1,26,29). Interestingly, among the progeny of Z-3651 *nit1* × Z-3654 NitM, differences at two *vic* loci were sometimes required for complete vegetative incompatibility. This finding suggests that differences at *vic* loci may be additive in terms of killing reaction, as has been reported for *Cryphonectria parasitica* (1) and *A. nidulans* (10).

Sexual recombination could be a useful tool for improvement of industrial strains of *G. zeae*. For example, we successfully crossed strain Z-4218, which is a type B strain that can be used in the zearanol production process (Table 5). Zearanol production could be higher in recombinants between different type B strains. Sexual recombination also provides a means to combine favorable mutations from "mutate and select" screening programs and to reduce the number of rounds of mutagenesis to which a strain is exposed. Sexual recombination also could be a valuable supplement to transformation, which has already become a powerful genetic tool in this fungus (36,39,47). Crossing could move transgenes into different backgrounds once they have been introduced into the organism, because repeat-induced point mutation (RIPing) is not known in *Fusarium* spp. (24). Backcrossing transformed strains could help remove deleterious mutations and aid in the selection of genetically stable laboratory and industrial strains.

Methods were developed for routine crossing of *G. zeae.* Both the mycelial plug and the mixed-inoculum methods were good for initiating crosses. The mycelial plug method was easiest, but the proportion of heterozygous perithecia was usually highest near the colony interface (data not shown). The mixed-inoculum method was more laborious, but gave a more uniform distribution of heterozygous perithecia across the plate. The spermatization method was least effective, possibly because timing and spore concentrations were not optimized. The method might be useful when it is important to control which parent serves as the female.

Both the cirrhus method and the ascospore print method were effective for detecting successful crosses. The cirrhus method was laborious, but allowed determination of segregation ratios from individual perithecia. One potential problem was contamination of cirrhi by airborne ascospores from elsewhere on the petri plate. For some genetic studies, alternative methods might be needed to eliminate this contamination by the parental types. The ascospore print method allowed quick and easy visualization of the pattern of heterozygous perithecia on carrot agar plates. It was very powerful for testing interfertility, because all sporulating perithecia on a plate could be assayed simultaneously. It might be useful for crossing industrial strains, because the recombinants are easily obtained and are wild type for nitrate metabolism.

G. zeae is homothallic; therefore, genetic markers are essential for distinguishing homozygous and heterozygous perithecia. Color mutants, antibiotic resistance mutants, and various auxotrophic mutants have been used as markers in other homothallic fungi (16, 32,34,35). The *nit* mutants were excellent genetic markers for several reasons. First, *nit* mutants are easily obtained in most *G. zeae* strains by positive selection on media amended with chlorate (5). We also obtained *nit* mutants from strains of *F. acuminatum*, *F. avenaceum*, *F. crookwellense*, and *F. culmorum* for the first time,

thereby extending the number of *Fusarium* spp. for which this technique has been shown to work to at least 10. Second, four classes (*nit1*, *nit3*, NitM, and *nnu*) of *nit* mutants can be obtained that can be easily distinguished on phenotyping media. These mutant classes do not crossfeed on agar media. If genetic linkage to a particular *nit* locus is undesirable, an alternative *nit* locus can be used. Third, progeny from crosses between classes segregate to produce 25% wild-type recombinants that are easily recognized on media containing nitrate as the sole nitrogen source. The easy recovery of wild-type recombinants is a potential advantage over methods that use antibiotic resistance or color mutant selectable markers. Fourth, *nit* mutants are usually stable in culture. In the course of this study, one unstable mutant strain was identified and discarded. However, the reversion rate for typical strains was estimated to be less than 10^{-5} per ascospore, which was negligible in these studies.

One potential problem is that nonrecombinant progeny from attempted crosses between strains in the same VCG may complement through somatic fusion on the MMTS plates when colonies are closely spaced. These complementing heterokaryons can be mistaken for wild-type sexual recombinants. This problem can be avoided by checking vegetative compatibility of parents or by dilution plating of ascospores to obtain well-separated colonies.

No fertility barriers were detected in a diverse set of *G. zeae* group 2 strains from maize, wheat, or barley from Japan, Nepal, and South Africa, and Indiana, Kansas, and Ohio in the United States (Tables 4 and 5). Strains of type A and B sensu Cullen et al. (13) crossed readily, showing that these strain types do not represent genetically isolated subpopulations. Several strains had reduced female fertility. Strain Z-4838 produced very few fertile perithecia. Strains Z-5048 and Z-4218 produced only infertile perithecia or no perithecia in our experiments. Nevertheless, these three strains were capable of functioning as males in some crosses, clearly placing them in group 2. In contrast, none of the group 1 strains was fertile as female or male in any crosses. The lack of interfertility between the two groups supports the suggestion by Burgess et al. (8) that group 1 should be given species rank. However, interfertility between groups needs to be retested with a larger collection of group 1 strains. Restriction fragment length polymorphism and RAPD studies suggested that *G. zeae* group 2 has greater relatedness with *F. culmorum* and *F. crookwellense* than with *G. zeae* group 1 (8,41). However, six strains of each of these two species were not interfertile with three strains of *G. zeae* group 2 (Table 5). Therefore, the taxon *G. zeae* group 2 sensu Burgess et al. (9) appears to circumscribe a potentially interbreeding biological species.

G. zeae group 2 is an unusual member of the genus *Gibberella.* It appears to be the only one in which ascospores are an epidemiologically important source of inoculum (11). It is one of only two *Gibberella* spp. reported to be homothallic (4). Presumably, heterothallism is the ancestral state in *Gibberella* spp. Since ascospores are important as primary inoculum, homothallic strains would have an advantage over heterothallic strains if suitable mates were not readily available. By becoming homothallic, *G. zeae* has maximized inoculum production without giving up sexual recombination ability as can happen in some heterothallic species (25).

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