Barrage Zone Formation Between Vegetatively Incompatible *Fusarium graminearum* (*Gibberella zeae*) Isolates

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ABSTRACT

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Vegetative compatibility has been used to assess the population biology of many fungal plant pathogens. However, for many species, including *Fusarium graminearum*, this has meant making auxotrophic mutants to force heterokaryon formation. A method was developed to observe barrage zones of thick, raised mycelium at the junctions of vegetatively incompatible *F. graminearum* isolates. The appearance of the barrage zones was influenced by the growth medium and the light. Barrage zones on V8 agar were thicker and better defined than those on potato dextrose agar, Spezieller Nährstoffarmer agar, and water agar. The addition of ground wheat kernels to V8 agar enhanced barrage zone formation. Incubating the cultures under constant light at 2,150 lx produced more distinct

barrage zones than constant light at 3,400 lx, constant darkness, or ambient room light. Forty-three *F. graminearum* isolates from 34 vegetative compatibility groups, determined previously using *nit* auxotrophic mutants, were paired in all combinations using these optimized conditions. Isolates in different vegetative compatibility groups typically formed distinct, thick barrage zones at their junctions. Pairs of isolates in the same vegetative compatibility group had a very slight or no visible reaction, or rarely, a distinct "line gap" of sparse mycelium. Subcultures from the same isolate typically had no visible reaction at their colony junctions; however, subcultures from some isolates had thin, slight barrage zones. This method was used to identify the proportion of each of four *F. graminearum* isolates from infected barley spikes in the field, inoculated previously with a mixture of conidia from these four isolates. Barrage zone formation represents a rapid method to screen vegetative compatibility groups in *F. graminearum* and may be useful for other *Fusarium* species.

Fusarium head blight (FHB) has caused severe losses in barley and wheat in the central Great Plains of North America on an annual basis since 1993. In Manitoba, Canada, *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch] was the most common *Fusarium* species isolated from barley spikes with symptoms of FHB collected from 1995 to 1998 (38). Other *Fusarium* species isolated from these spikes included *F. poae*, *F. sporotrichioides*, and *F. avenaceum* (38). The proportion of *F. graminearum* relative to other *Fusarium* species isolated from FHB-infected kernels on barley increased from 74% in 1995 to 96% in 1998 (38). The increase in *F. graminearum* in barley since 1993 has been accompanied by higher FHB severities and associated yield losses, but more critically, increased the levels of deoxynivalenol (DON) in infected grain, rendering it unusable for malting or animal feed (27).

In many species of filamentous fungi, physiologically distinct individuals can fuse asexually to form a stable heterokaryon (23). The individuals that can fuse and form stable heterokaryons are said to be vegetatively compatible and therefore belong to the same vegetative compatibility group (VCG). Vegetative compatibility has been studied in many fungal genera including *Podospora*, *Aspergillus*, *Cryphonectria*, and *Neurospora* (22). Genetic control of vegetative compatibility was found to be conditioned by numerous loci in those species where it has been investigated (3). Incompatibility can be triggered by either allelic or nonallelic interaction between those loci. VCGs have been used to study the population biology of many *Fusarium* species including *F. oxysporum*, *F. moniliforme* (22), *F. poae* (24), and *F. avenaceum* (34).

VCG diversity is very high within *F. graminearum* even at the local level. Twenty-four different VCGs were found among 24 isolates from different wheat fields in Kansas (4) and 19 VCGs were found among 26 isolates sampled from wheat spikes in a 0.25-m² section of a single field (5), indicating that the *F. graminearum* population from wheat is genetically highly variable even within a very small area. The *F. graminearum* population from barley also has a high VCG diversity. There were 21 VCGs among 22 *F. graminearum* isolates from different barley fields in Manitoba and 16 VCGs among 21 isolates from four barley fields that were sampled more repeatedly at a local level (26).

In *Fusarium* spp., VCGs have been determined primarily through direct heterokaryon formation using auxotrophic mutants such as nitrate nonutilizing (*nit*) mutants (22). The technique, developed by Puhalla (33), selects for spontaneously occurring *nit* mutants on chlorate containing medium and has been an effective method to characterize VCGs in many fungal species (22). Although this technique can be used to force and detect heterokaryon formation, the generation and storage of *nit* mutants is time consuming. Nitrate nonutilizing mutants are also difficult to generate from some species such as *Sclerotinia sclerotiorum* that have a level of natural resistance to chlorate (20).

Vegetative compatibility can be assessed using mycelial interactions or barrage zone formation in the absence of forcing auxotrophic markers in other fungal species. Barrage zone formation is well described in fungal species such as *Cryphonectria parasitica* (2), *Podospora anserina* (3,15), and *Ophiostoma ulmi* (7). Vegetatively incompatible isolates form a barrage zone at the line of junction of the two colonies, whereas compatible strains do not form barrage zones and typically grow into each other without any morphological differentiation. The description of the barrage zone, or antagonistic interaction, differs between species and various types of barrages have been described for some species such as *O. ulmi* (7). Some common characteristics of barrage zones are

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a central region of dead or dying cells, a darkly pigmented area, and a higher and thicker mycelial layer (22). Mycelial incompatibility in *S. sclerotiorum* (20) and *Sclerotium cepivorum* (14) was described as a distinct reaction line with abundant aerial mycelium or alternatively as a zone of thin mycelium. Kohn et al. (21) defined mycelial incompatibility, evidenced by the formation of a reaction line between strains in pairings on agar medium, as the inability of two strains to fuse and form one colony. Mycelial compatibility was described as one of several events associated with vegetative compatibility, the ability of two strains to fuse and form a stable heterokaryon (21). Ford et al. (16) used auxotrophic mutants to force heterokaryon formation in *S. sclerotiorum* and found that VCGs matched mycelial compatibility groups for five of the six isolates that were used, but did not match for the sixth isolate.

Barrage zones in *Fusarium* spp. were originally described for *F. oxysporum* f. sp. *niveum* where it was termed "cultural interaction" and described as a white band of raised dense mycelium that developed between wild-type cultures and mutants that arose during culturing on media (29). Cultural interaction was also described in *F. oxysporum* f. sp. *cubense* by Stover (37) as raised white bands of dense mycelium developed between some colonies but not between others. The author designated two types of isolates: interactants type I and type II. An interaction zone was observed at the junction of interactant I and II colonies but not between colonies of the same interactant type. This phenomenon also was investigated by Page (31) who paired six *F. oxysporum* f. sp. *cubense* hyphal tip subcultures from the same original isolate. Because cultural interaction occurred between all thalli, which were thought to be genetically identical, Page (31) concluded that cultural interaction could not be used to diagnose different variants of this fungus.

We noticed slight raised zones of dense white mycelium at the junction of most *F. graminearum* isolates from FHB-infected barley kernels growing on potato dextrose agar (PDA) (Acumedia, Baltimore, MD) but some pairs had no noticeable zone of interaction. If barrage zone formation was correlated with vegetative incompatibility in *F. graminearum*, it could be a more rapid, less labor-intensive method to determine VCGs than the use of *nit* mutants.

The objectives of this study were to develop a reliable system to visualize barrage zones in *F. graminearum* and to determine if these zones were indicative of vegetative incompatibility. To optimize the conditions for barrage zone production in *F. graminearum* we tested an array of media, media supplements, and lighting conditions. We screened these conditions first on a selected subset of vegetatively incompatible isolates. Once the optimal conditions for barrage zone formation were determined, we tested the relationship between barrage zones and vegetative compatibility groupings based on forcing heterokaryon formation using auxotrophic *nit* mutants. Finally, we demonstrated how barrage zone formation can be used to identify *F. graminearum* isolates.

MATERIALS AND METHODS

Influence of growth medium and light on barrage zone formation. Various nutrient and light combinations were tested to determine which set of conditions would produce the thickest and most well-defined barrage zones between *F. graminearum* isolates. The media tested initially included PDA, Spezieller Nährstoffarmer agar (SNA [8]: KH_2PO_4 , 1.0 g; KNO_3 , 1.0 g; MgSO_4 , 0.5 g; KCl , 0.5 g; glucose, 0.2 g; sucrose, 0.2 g; and agar, 20.0 g per liter of medium), water agar (20.0 g of agar per liter), V8 agar I (150.0 ml of V8 juice [Campbell Soup Company, Toronto, ON, Canada], 15.0 g of CaCO_3 , 20.0 g of agar, and H_2O to 1.0 liter), V8 agar II (300.0 ml of V8 juice, 15.0 g of CaCO_3 , 20.0 g of agar, and H_2O to 1.0 liter), V8 agar III (150.0 ml of V8 juice, 20.0 g of

agar, and H_2O to 1.0 liter), and V8 agar IV (300.0 ml of V8 juice, 20.0 g of agar, and H_2O to 1.0 liter). To determine the influence of these different media formulations on the thickness and definition of barrage zone development, five *F. graminearum* isolates, originally isolated from barley kernels, were selected. Each of these *F. graminearum* isolates was in a different VCG as determined by direct observation of heterokaryon formation using *nit*-nonutilizing mutants (26). The five isolates were each placed onto five petri dishes (9 cm) for all of the media listed above and incubated for 7 days under constant light (2,150 lx). Four isolates were placed equidistantly around the outer edge of each plate and the fifth isolate was placed in the center of the dish. A different isolate of the five was placed in the center of each of the five dishes per media tested. The relative size and distinctiveness of the barrage zones formed between the isolates on the different media were compared visually after 7 to 14 days incubation.

Because light also appeared to have a pronounced effect on the expression of barrage zone formation, several levels were tested: (i) 3,400 lx continuous light, $23 \pm 2^\circ\text{C}$; (ii) 2,150 lx continuous light, $23 \pm 2^\circ\text{C}$; (iii) approximately 750 lx light during the day (10 h) and 0 lx at night (14 h), $21 \pm 2^\circ\text{C}$; and (iv) 0 lx continuous darkness, $21 \pm 2^\circ\text{C}$. Light was supplied by cool white fluorescent bulbs. The effects of these different light levels were tested in combination with various modifications to V8 agar III. These included the addition of ground wheat, oat, or barley kernels (kernels from a mixture of cultivars were used for these cereals and they were ground in a coffee grinder) at 2.5, 5.0, 10.0, and 20.0 g/liter and a multivitamin solution (11) at 5, 10, 15, 20, 25, and 30.0 ml/liter. In total there were four light conditions and 18 media resulting in 72 different media and light combinations. The same five *F. graminearum* isolates were tested in each combination of medium and light regime previously described. The relative size and distinctiveness of the barrage zones formed between isolates under the various growth conditions were compared visually at 7 to 14 days.

The relationship between barrage zone and vegetative compatibility. To determine if barrage zone formation was indicative of vegetative incompatibility, 43 *F. graminearum* isolates belonging to 34 VCGs, determined previously by direct heterokaryon complementation using *nit* mutants (26), were paired in all possible combinations on V8-wheat medium (150 ml of V8 juice, 20 g of ground wheat seed, and 20 g of agar per 1 liter of medium). Four isolates were placed equidistantly around the outer edge of a petri dish filled with V8-wheat medium and one isolate was placed in the center. The petri dishes were incubated at room temperature ($23 \pm 2^\circ\text{C}$) under constant light (approximately 2,150 lx). Each isolate was paired with every other isolate and itself at least twice. The presence or absence of barrage zones at the junctions of growing colonies was recorded after 7 to 14 days incubation.

Using barrage zones to identify *F. graminearum* isolates. Cultures of four isolates of *F. graminearum* (G1, G2, G3, and G6), each previously determined to belong to a different VCG (26), were grown on PDA. After 10 days, strips of mycelium and agar were cut from each isolate and added in equal proportions to 1 liter of liquid medium (CMC medium [9]: NH_4NO_3 , 1.0 g; KH_2PO_4 , 1.0 g; MgSO_4 , 0.5 g; yeast extract, 1.0 g; and carboxymethyl cellulose, 15.0 g per liter of medium). The mixed culture was grown for 7 days, with constant aeration from a compressed air line, and then filtered through cheesecloth. The conidia were counted with a hemacytometer, and the conidial concentration was adjusted to 5×10^4 conidia per ml. This mixture of conidia was used to inoculate field plots comprised of one 1.5 m row for each of the four barley cvs. AC Lacombe, AC Oxbow, Argyle, and Bedford with 30 cm spacing between the rows at Morden, Manitoba in 1997. The experiment was randomized with two replicates. At heading, 1.0 liter of the conidial suspension was sprayed onto the barley spikes of each of the four rows per plot and each four-row plot was covered with a plastic tent overnight to main-

tain high humidity. At maturity, the spikes were harvested and threshed. One hundred kernels per row were subsequently surface-sterilized for 3 min in 0.3% NaOCl, allowed to dry on filter paper, and placed in petri dishes filled with PDA. Any *F. graminearum* colonies that grew out from the seeds were subcultured and transferred to the center of a petri dish containing V8-wheat medium with the original tester isolates G1, G2, G3, and G6 placed throughout the petri dish to test for the development of barrage zones. The four tester isolates were placed on the petri dishes with four isolates recovered from the colonized barley kernels (unknown isolates) in such a manner that barrage zones could be observed between the four unknown isolates and each of the four tester isolates. The plates were incubated at room temperature under constant light (2,150 lx) for 7 to 10 days. Twenty additional *F. graminearum* isolates, recovered from naturally FHB-infected barley kernels from plots adjacent to the inoculated plots, also were tested for barrage zone formation with the tester isolates to determine if the VCGs represented by the tester isolates occurred in the local *F. graminearum* population.

To determine the relative production of conidia among the four tester isolates (G1, G2, G3, and G6), each isolate was grown separately in 1-liter flasks of CMC media for 7 days as described previously. These cultures were filtered through cheesecloth, and the conidia were counted with a hemacytometer to determine the number of conidia produced per milliliter of liquid media for each isolate.

RESULTS

Influence of growth medium and light on barrage zone formation. Both the growth medium and light affected formation of barrage zones between *F. graminearum* isolates. Among the media first tested (PDA, SNA, water agar, V8 agar I, V8 agar II, V8 agar III, and V8 agar IV), the most distinct, dense, and largest barrage zones were produced on V8 agar III. The addition of ground wheat, barley, or oat kernels, or a vitamin solution generally resulted in larger, more well-defined barrage zones. Ground wheat at the rate of 20 g/liter was the most effective supplement to V8 agar III for enhancing the size, density, and distinctiveness of the barrage zones under all light conditions tested. Therefore, the optimal medium for distinguishing barrage zones, among

those tested, was V8-wheat media (150 ml of V8 juice, 20 g of ground wheat, and 20 g of agar per liter of medium).

Light also influenced barrage zone formation. Cultures grown under constant light of 2,150 lx had larger, more distinctive barrage zones than cultures grown under other light conditions, regardless of the medium used. Distinctive barrage zones could be seen on most of the media when incubated under constant light of 2,150 lx but only on the V8-wheat medium under other light conditions (Fig. 1). Cultures grown on V8-wheat medium under constant light of 2,150 lx produced barrage zones with thick, raised mycelium, approximately 5 to 7 mm in width, at the lines of junction between two vegetatively incompatible *F. graminearum* isolates. No barrage zone, or a very slight zone, was observed at the junction of two colonies subcultured from the same original isolate (Fig. 2). This combination of medium (V8-wheat) and light (2,150 lx) was used for the subsequent barrage zone tests.

The relationship between barrage zone and vegetative compatibility. The presence or absence of barrage zone formation was observed in more than 1,849 pairings between 43 *F. graminearum* isolates in all possible two-way combinations. When isolates of *F. graminearum* in different VCGs were paired there was typically a thick barrage zone between colonies (Fig. 2); however, some variation in the thickness and abundance of aerial mycelium within the barrage zone occurred between different isolates. Certain isolates formed smaller, weaker barrage zones with all other isolates with which they were paired, while some pairs of isolates had smaller, weaker-than-average barrage zones between them. When subcultures taken from the same isolate were paired, there typically was no visible barrage zone at the line of junction of two colonies that just grew into each other without a line of demarcation. In some self-pairings, however, a slight barrage was noted (Fig. 2, between A and A), although the barrage zone was smaller in width and had less aerial mycelium than those between vegetatively incompatible isolates. In nearly all pairings between different isolates in the same VCG there was either no barrage

Fig. 1. Effects of the combination of light and medium composition on barrage zone formation in *Fusarium graminearum*. The media were V8, V8 plus vitamins, V8 plus barley, and V8 plus wheat, and incubation was under one of the following light regimes: (i) 3,400 lx, (ii) 2,150 lx, (iii) approximately 750 lx light during the day and 0 lx at night, or (iv) 0 lx.

Fig. 2. Four *Fusarium graminearum* isolates A, B, C, and D plated onto V8-wheat medium. Isolates A and C were in the same vegetative compatibility group and B and D were in different groups as determined by direct heterokaryon formation. Fully incompatible barrage reactions, characterized by a thick line of raised mycelium, occurred between isolates A and B, B and C, A and D, and C and D. The two subcultures of isolate A had a very slight reaction between them typical of some self-self pairings. Isolate A formed a "line gap" reaction with isolate C, characterized by a line of sparse mycelium between the two isolates.

Using barrage zones to identify isolates. Isolates G1, G2, G3, and G6 of *F. graminearum* previously were assigned to different VCGs based on complementation of *nit* mutants (26). When tested using the barrage zone protocol, barrage zones formed at the junction of all pairings between these isolates but not between subcultures from the same isolate. This technique was used to rapidly identify *F. graminearum* isolates recovered from barley previously inoculated with a mixture of these four tester isolates (Fig. 3). The proportion of each tester isolate in the inoculated spikes was estimated based on barrage zone formation between the isolates recovered from the infected barley kernels and the four tester isolates (Table 1). All *F. graminearum* isolates from the inoculated plants formed barrage zones with three of the four tester isolates (indicating incompatibility), but not with the fourth tester isolate (indicating compatibility). In total, 48 isolates were compatible with G1 (59%), 22 with G2 (27%), 0 with G3, and 12 with G6 (15%). These proportions approximate the relative ratio of conidial production of these isolates (G1:G2:G3:G6 = 49:29:1:20) when subsequently grown individually in separate flasks of CMC medium. Thus, the ratio of the isolates recovered from the infected seed probably reflects relative differences in conidial production in the mixed culture rather than differences in relative aggressiveness of the isolates.

A top-down view of a circular petri dish containing a grid of agar blocks. The blocks are separated by white lines. The growth of *Aspergillus fumigatus* is visible on the agar blocks, with labels G1, G2, G3, and G6 indicating different growth stages or conditions. Specific areas of growth are marked with letters a, b, c, and d. The growth appears as a dense, fuzzy, brownish-green mass on the agar blocks.

from the inoculated barley spikes were compatible with one of the four tester isolates indicates that the isolates recovered from the inoculated plots probably originated from the inoculation with the mixture of tester isolates and not from naturally occurring *F. graminearum*.

A method was developed to observe barrage zones between vegetatively incompatible *F. graminearum* isolates. The thickness of these zones and the abundance of aerial mycelium was influenced by the growth medium used and the light intensity. The optimal conditions were observed on V8-wheat media with cultures grown under constant light of 2,150 lx. Optimizing the growth medium also was important for the detection of barrage zones in *Leucostoma persooni* (1) and *Diaporthe phaseolorum* (32), and both growth media and light influenced barrage zone formation in *Monilinia fruticola* (36).

Using these optimized conditions, 43 *F. graminearum* isolates belonging to 34 VCGs, defined using *nit* mutants, were paired in all combinations to observe the presence or absence of barrage zones between them. In all cases, a barrage zone formed between vegetatively incompatible isolates, although the width and thickness of these zones varied somewhat depending on the isolates involved. Brasier (7) described four types of barrage zones between vegetatively incompatible isolates of *O. ulmi*, differentiated primarily on the thickness of the barrage zone and the restriction of cytoplasmic transfer. Brasier (7) determined that the thickness of the barrage zone was correlated with the number of vegetative incompatibility genes that were different between the isolates. The thickness of the barrage zone also was specific to the isolates paired in *C. parasitica* (2). The genetics of vegetative compatibility in *F. graminearum* have not been investigated thoroughly but Bowden and Leslie (6) found evidence for at least two or three vegetative incompatibility genes, segregating in the two crosses analyzed. Further research is needed to determine the genetic control of thickness of barrage zones in *F. graminearum*.

When subcultures of the same isolate were paired, typically no visible barrage zone at the line of junction of two colonies was observed that grew into each other with no line of demarcation. In some self-pairings a slight barrage was noted, but it was much thinner with less aerial mycelium than the barrage zones between vegetatively incompatible isolates. Self-incompatibility, detected using *nit* mutants, has been described for some isolates of *F. moniliforme* (12) and *F. oxysporum* (17). Although all of the *F. graminearum* isolates used in our study were self-compatible using *nit* mutants (26), there may be some degree of slight self-incompatibility, indicated by thin barrage zones, in some isolates.

Barley cultivar	Replicate	<i>F. graminearum</i> isolate				
		G1	G2	G3	G6	Total
Argyle	1	3	4	0	3	10
Bedford	1	7	3	0	0	10
AC Lacombe	1	5	3	0	1	9
AC Oxbow	1	5	2	0	3	10
Total	1	20	12	0	7	39
Argyle	2	11	4	0	3	18
Bedford	2	6	3	0	0	9
AC Lacombe	2	6	3	0	1	10
AC Oxbow	2	5	0	0	1	6
Total	2	28	10	0	5	43
Grand total	1 and 2	48	22	0	12	82
Average (%)	1 and 2	58.5	26.8	0	14.6	...

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The barrage zones that Page (31) observed between hyphal tip subcultures from the same original *F. oxysporum* f. sp. *cubense* isolate could represent a similar type of slight self-incompatibility. However, the barrage zones observed between the two interactant types of *F. oxysporum* f. sp. *cubense* by Stover (37) appear to be similar to those between vegetatively incompatible *F. graminearum* isolates.

In the majority of pairings between different isolates in the same VCG there was no barrage zone or a very slight zone. With a very few pairs of vegetatively compatible isolates, a small, distinct, nearly clear zone (2 to 4 mm) formed with little or no visible mycelium between the colonies (Fig. 2, between C and A), similar in appearance to the line gap reaction in *O. ulmi* (7). This reaction was not observed in any self-pairings nor in any pairings between vegetatively incompatible isolates, as defined using *nit* mutants (26). Brasier (7) described pairs of *O. ulmi* isolates that form a line gap as differing at only one vegetative incompatibility locus and thought this reaction would not limit cytoplasmic transfer. It is interesting that in our study the only pairs of *F. graminearum* isolates to form the line gap reactions were from the same VCG, as determined by *nit* mutants. It therefore appears that the line gap reaction does not interfere with the successful establishment of a heterokaryon between these pairs of isolates on nitrate medium.

There appears to be a close relationship between vegetative compatibility and barrage zone formation within *F. graminearum*. Since the *F. graminearum* population is extremely diverse for VCGs (4,26), even at the local level (5,26), the VCG of an isolate may be nearly as useful as a genetic fingerprint for genotype identification. However, genetic diversity within and between VCGs in *F. graminearum* needs to be investigated as has been done in other *Fusarium* species (10). Zeller et al. (40) compared 26 *F. graminearum* isolates from 10 wheat heads in a 0.5 × 0.5 m quadrant of a wheat field for both VCGs using *nit* mutants and amplified fragment length polymorphism (AFLP) markers. High levels of genotypic variability were revealed by both methods. The same sets of isolates were identified as probable clones using both methods, although isolates in the same VCG could vary at one or two AFLP loci (40). Population analysis of *F. graminearum* would be greatly facilitated by a rapid method for determining the VCG of an isolate that does not involve generating and screening auxotrophic mutants. Barrage zone formation is useful as a rapid indication of VCG to distinguish the component isolates in an *F. graminearum* inoculum mixture, provided the isolates in the mixture are in different VCGs, as demonstrated in our study. It was also useful to distinguish exogenously applied *F. graminearum* from natural background *F. graminearum*. A similar trial with field-grown corn infected by *F. moniliforme* used *nit* mutants to distinguish applied inoculum from background *F. moniliforme* (30). Barrage zone formation should facilitate the study of cultivar by isolate interactions because the relative aggressiveness of isolates in a mixture can be assessed directly by determining the proportion of each isolate in infected seed after inoculation with equal portions of conidia from two or more isolates. Factors such as the relative growth and conidiation rates of the isolates in the mixture also will need to be considered.

Because *G. zeae* (*F. graminearum*) is homothallic, a genetic marker system is necessary to distinguish hybrid from selfed perithecia when studying crosses. Bowden and Leslie (6) used *nit* mutants as parents and identified hybrid perithecia by segregation for nitrate utilization. A genetic map for *G. zeae* (*F. graminearum*) was generated by crossing complementary *nit* mutants from two different strains and selecting the hybrid progeny with the ability to utilize nitrate (18). The progeny analyzed to generate the map were therefore a subset, selected for nitrate utilization, of the total pool of hybrid progeny, which may have led to segregation distortion in some regions of the map (18). Barrage zone formation could be used to identify hybrid perithecia with-

out the need to create *nit* mutants from the parental strains and without selection in the progeny. If two isolates in different VCGs are paired, no progeny from a selfed perithecium should form a barrage zone with the parental isolate but should form a barrage zone with the nonparent, whereas progeny from a hybrid perithecium should segregate for barrage zone formation with one or the other parent and should contain some ascospore progeny that would form barrage zones with both parents. This assumes that all ascospores from a single perithecium are derived from a single fertilization event. A similar strategy was proposed for crossing the homothallic ascomycete *C. parasitica* (2). Anagnostakis (2) also suggested that the number of vegetative incompatibility loci for which the paired isolates differed could be determined directly by pairing progeny isolates to a single parental tester. If the frequency of the single parental tester types in the hybrid progeny is approximately 50%, this suggests that the two parents differed at one locus, 25% suggests two loci, etc.

The high level of genetic diversity within *F. graminearum* for both VCGs (4,5,26) and molecular markers (13,35,39,40), and the indication that both conidia and ascospores play a role as inoculum sources for FHB (25), suggest a relatively high level of outcrossing in *G. zeae* (*F. graminearum*). Estimating the actual level of outcrossing in natural populations is important for understanding and controlling this important plant pathogen. Barrage zone formation would facilitate the determination of the naturally occurring rate of outcrossing, because hybridization could be determined quickly by looking for barrage zone formation between the ascospore progeny from a single perithecium. Sibling progeny from perithecia produced by outcrossing should segregate for the formation of thick barrage zones at the junctions of their colonies, whereas sibs from selfed perithecia should not form thick barrage zones at the junctions of their colonies. Milgroom et al. (28) found that vegetative compatibility could be used to detect outcrossing in natural populations of *C. parasitica* but it was not as efficient as DNA markers. From a sample of 20 perithecia, VCGs only detected 11 as outcrossing compared with 16 detected as outcrossing with DNA markers. However, given the relatively high level of naturally occurring VCG diversity in *F. graminearum* (4,5,26) and the close relationship between VCG diversity and genetic diversity (40), barrage zone formation may be useful to rapidly detect outcrossing in *G. zeae* (*F. graminearum*). Bowden and Leslie (6) found a maximum of 35% hybrid perithecia in controlled *G. zeae* (*F. graminearum*) crosses. If the outcrossing rate is very low in natural populations of *G. zeae* (*F. graminearum*), then a large number of perithecia will need to be screened to detect the hybrids and get a reliable estimate of their frequency. Barrage zone formation should be relatively quick and more efficient than using molecular techniques or conventional auxotrophic mutant pairings because the ascospore progeny can be cultured and paired directly.

Members of the same VCG in *F. oxysporum*, defined using *nit* mutants, normally have similar multilocus haplotypes and belong to the same clonal lineage (19). The VCG of an *F. oxysporum* isolate is therefore very informative because the isolate is likely to be genetically similar to other members of that VCG. It may be possible to observe barrage zone formation between vegetatively incompatible *F. oxysporum* isolates if the cultural conditions are optimized for this species. Barrage zones in *F. oxysporum* could have already been observed by Stover (37). It might also be possible to observe barrage zone formation in other *Fusarium* species, although the optimal cultural conditions for the formation of barrage zones could be unique for each species.

A reliable technique was developed to produce barrage zones at the junctions of vegetatively incompatible *F. graminearum* isolates. The presence or absence of barrage zones between both vegetatively incompatible and vegetatively compatible isolates demonstrated that barrage zone formation is closely correlated with vegetative incompatibility determined using *nit* mutants.

This technique was used to identify isolates from barley spikes inoculated in the field and there are many other potential applications.

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