

Influence of the Host Contact Sequence on the Outcome of Competition among *Aspergillus flavus* Isolates during Host Tissue Invasion[∇]

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Biological control of aflatoxin contamination by *Aspergillus flavus* is achieved through competitive exclusion of aflatoxin producers by atoxigenic strains. Factors dictating the extent to which competitive displacement occurs during host infection are unknown. The role of initial host contact in competition between pairs of *A. flavus* isolates coinfecting maize kernels was examined. Isolate success during tissue invasion and reproduction was assessed by quantification of isolate-specific single nucleotide polymorphisms using pyrosequencing. Isolates were inoculated either simultaneously or 1 h apart. Increased success during competition was conferred to the first isolate to contact the host independent of that isolate's innate competitive ability. The first-isolate advantage decreased with the conidial concentration, suggesting capture of limited resources on kernel surfaces contributes to competitive exclusion. Attempts to modify access to putative attachment sites by either coating kernels with dead conidia or washing kernels with solvents did not influence the success of the first isolate, suggesting competition for limited attachment sites on kernel surfaces does not mediate first-isolate advantage. The current study is the first to demonstrate an immediate competitive advantage conferred to *A. flavus* isolates upon host contact and prior to either germ tube emergence or host colonization. This suggests the timing of host contact is as important to competition during disease cycles as innate competitive ability. Early dispersal to susceptible crop components may allow maintenance within *A. flavus* populations of genetic types with low competitive ability during host tissue invasion.

Aspergillus flavus is the primary causal agent of aflatoxin contamination in food and feed crops, including maize, cotton, peanuts, and tree nuts (3, 14, 38). Aflatoxins are highly carcinogenic mycotoxins, and aflatoxin levels in crops for human or animal consumption are tightly regulated in many countries, including the United States (12). Aflatoxin contamination results from crop infection by *A. flavus* communities composed of genetically diverse vegetative compatibility groups (VCGs) (2, 22). VCGs vary in several characteristics, including the ability to produce aflatoxins (3, 35, 38). Aflatoxin-producing ability is not correlated with either virulence to plant hosts (7) or competitiveness during invasion of host tissues (30). Crop aflatoxin content is dictated in part by the aflatoxin-producing potential of crop-associated *A. flavus* communities (9, 21, 24) and, presumably, by isolate-isolate interactions on individual crop components (12, 30). Atoxigenic biocontrol strains of *A. flavus* have been deployed commercially to reduce aflatoxin contamination of crops through competitive displacement of aflatoxin producers (12, 15). Although variation among *A. flavus* isolates in competitive ability during plant tissue invasion is known (30), factors influencing the outcome of competition during host infection are not fully understood.

Organisms utilized for biological control of microorganisms by competitive exclusion generally require prior host contact to achieve optimal efficacy. For example, nonpathogenic *Fusar-*

ium oxysporum isolates reduce the severity of *Fusarium* wilts and root rots by competition for nutrients and infection sites as well as induced host resistance (5, 18, 28); all three modes of action require that the biocontrol agent contact the host prior to the pathogen. In addition to direct antagonism through production of antibiotics, biocontrol bacteria prevent fire blight (causal agent, *Erwinia amylovora*) and crown gall (causal agent, *Agrobacterium tumefaciens*) through physical occupation of infection courts and competition for nutrients (25, 29, 40, 42). When pathogen exclusion relies on competition for a niche (i.e., physical space or a specific nutrient source), both inoculum density and timing of application influence efficacy (5, 28, 42). Reductions in crop aflatoxin content, for example, are significantly greater when the biocontrol *A. flavus* is applied to the crop 24 h prior to an aflatoxin producer than with simultaneous inoculation of atoxigenic and aflatoxin-producing isolates (6, 8, 11).

Initial host contact and attachment to susceptible plant parts is critical for virulence of many plant pathogens (17), and competition for a finite number of attachment sites is one mechanism by which a biocontrol organism may prevent both disease symptoms and toxin formation. Adherence of conidia may involve active or passive mechanisms. Active attachment requires metabolic energy and usually coincides with germination (26, 31, 41). Many conidia, however, passively attach to surfaces via preformed adhesives (20, 39), hydrophobic interactions (16, 20, 31, 39), or lectin-mediated binding (27). Attachment may be specific (e.g., lectins) or nonspecific (e.g., hydrophobic attachment), and attachment sites on the plant surface may be limited (28, 32, 36). The importance of host attachment to *A. flavus* virulence has not been explored. How-

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ever, initial host contact may influence competitive interactions by exclusion of subsequent isolates from attachment sites on the host surface.

Reductions in aflatoxin contamination following application of biocontrol strains either concurrently with or prior to aflatoxin-producing strains has been attributed to physical exclusion of aflatoxin producers in cotton (8) and maize (6, 30) and to competition for nutrients (10, 23). Competitive displacement by atoxigenic isolates has been quantified (10, 30), but factors contributing to success during competition are not known. The current study sought to determine the extent to which initial host contact confers competitive advantage during host infection.

MATERIALS AND METHODS

Fungal isolates and inoculum production. One isolate each from the VCGs CG136 (isolate 136), YV04 (isolate Red-4-E), MR17 (isolate MR2-17), and EB01 (E. Bernard B-c) were used in this study. All isolates with the exception of EB01 were from soil in Arizona; EB01 was isolated from Texas cottonseed. To verify VCGs and ensure the genetic purity of cultures, nitrate-nonutilizing auxotrophs (*niaD*⁻) were used. Previously, YV04 and EB01 were some of the most competitive and MR17 was the least competitive among isolates from 38 VCGs tested for the ability to compete with CG136 (30).

Isolates were stored on silica gel and cultivated on 5/2 agar (5% V8 juice, 2% agar, pH 5.2). Conidial suspensions were maintained in 4-ml vials by suspending agar plugs of mature cultures in sterile distilled water. Plates of 5/2 agar were centrally seeded with conidial suspensions and incubated at 31°C for 7 days. Conidia dislodged from plates with sterile cotton swabs were suspended in sterile distilled water, and the turbidity of conidial suspensions was measured (turbidimeter; Orbeco Analytical Systems, Farmingdale, NY). A turbidity-versus-CFU standard curve was used to estimate the number of conidia, and suspensions were adjusted to the appropriate concentrations (see below).

Maize inoculations. Intact, uniformly sized kernels of either Pioneer hybrid 33B50 or 33B54 grain were surface disinfected by submersion in 80°C water (45 s). Kernels (5 g) were added to sterile 250-ml Erlenmeyer flasks sealed with gas-permeable plugs (Bugstoppers; Whatman, Piscataway, NJ). Kernels were plated on agar to confirm viability (i.e., the ability of kernels to germinate) and a lack of contaminating fungi. The appropriate number of conidia (indicated below) was suspended in either half the volume of water required to bring the kernel moisture to 25% or, for mixed inocula, the entire volume of water. For example, if kernels were initially at 8% moisture, conidia in a total of 850 µl of water were added to 5 g kernels (850 µl mixed inocula or 425 µl [each] of the individual isolates). Moisture content of kernels after surface disinfection ranged from 6.6 to 10.6%. Kernels were coated with inoculum by gently shaking (for 10 s) flasks after adding conidial suspensions. All treatments had four replicates, and experiments were performed twice. Treatments were arranged in a randomized complete block design. Inoculated kernels were incubated at 31°C for 7 days and then dried at 60°C (48 h) to stop fungal growth.

Sequence of inoculation and inoculum concentration assays. The isolates CG136, YV04, and MR17 were coinoculated in pairs (5×10^4 conidia suspended in 425 µl water each) on kernels of Pioneer hybrid 33B50 grain, with each isolate added either 1 h prior to or 1 h following the other isolate. Additionally, isolate pairs were mixed and kernels were inoculated simultaneously with 5×10^4 conidia of each isolate suspended in 850 µl water (Table 1). In a separate experiment, the effect of inoculum concentration was tested; kernels were inoculated with either 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , or 10^7 conidia of YV04 followed by an equal quantity of CG136 conidia 1 h later.

Binding inhibition assay. Grain of Pioneer hybrid 33B50 became unavailable, so the closely related hybrid 33B54 was used in all subsequent experiments. To ensure results were similar between cultivars, competition between YV04 and CG136 on grain of hybrid 33B50 was compared to competition on 33B54. In an attempt to interfere with potential attachment to kernel surfaces by the first isolate, kernels were coated with dead conidia of isolate EB01 prior to inoculation with isolates YV04 and CG136. Isolate EB01 was selected because in a previous study, it was the most competitive among 38 isolates on maize when inoculated 1 h prior to CG136 (30), and it could be distinguished from isolates YV04 and CG136 with the pyrosequencing assay used in this study in order to confirm a lack of growth by EB01. EB01 conidia were killed aseptically by submersion in either 80% ethanol or 80°C water for 10 min or by passage through

TABLE 1. Influences of inoculation sequence on competitive abilities of *A. flavus* isolates coinfecting maize kernels

Sample type ^b	Isolate A	Isolate B	% isolate A DNA (SE) ^a		
			Isolate A first ^c	Isolate A and B, mixed ^d	Isolate B first ^c
Kernels	CG136	YV04	65 (4)* A, b	49 (4) B, b	29 (4)* C, b
	CG136	MR17	81 (2)* A, a	61 (4)* B, b	50 (4) C, a
	YV04	MR17	85 (4)* A, a	75 (5)* AB, a	62 (6) B, a
Conidia	CG136	YV04	74 (4)* A, b	50 (3) B, b	31 (4)* C, b
	CG136	MR17	90 (2)* A, a	72 (2)* B, a	50 (4) C, a
	YV04	MR17	92 (2)* A, a	68 (4)* B, a	54 (3) C, a

^a Proportion of total *A. flavus* DNA comprised of isolate A DNA. Means followed by the same letter are not significantly different by Fisher's LSD test. Capital letters before the comma indicate differences among inoculation sequence treatments (rows); lowercase letters following commas indicate differences among pairs of isolates (columns). Values followed by asterisks are significantly different from 50%.

^b *A. flavus* DNA from kernel-infecting hyphae and that from conidia from the surface of kernels were analyzed separately.

^c Kernels were inoculated with isolate A 1 h prior to inoculation with isolate B.

^d Conidia from isolates A and B were mixed prior to inoculation of kernels.

^e Kernels were inoculated with isolate B 1 h prior to isolate A.

a French pressure cell (Aminco, Silver Spring, MD). Ten milliliters of a dense suspension of conidia (approximately 10^8 CFU/ml) was passed through a French cell three times, and the resulting conidial fragments were filtered (0.80-µm pore size) to remove intact conidia. Treated conidia were tested for growth on 5/2 agar to confirm lack of viability.

In the first trial, kernels were coated with 250 µl of heat-killed conidia (4×10^7 conidia/ml) by gently shaking (for 10 s) flasks after adding the suspension. Conidia were stained with lactophenol cotton blue and observed microscopically to confirm their presence on kernel surfaces. One hour later, 5×10^4 conidia of YV04 in 239 µl water were added in a similar manner. After an additional hour, kernels were inoculated with 5×10^4 conidia of isolate CG136 in 239 µl water to bring the total moisture content to 25%. In the second trial, kernels were similarly coated either with heat-killed conidia, ethanol-killed conidia, or conidial fragments of EB01 (4×10^6 fragments/ml based on hemocytometer counts). Kernels were then inoculated with YV04, followed by CG136 1 h later. In both trials, control treatments included uncoated kernels inoculated with YV04 first, CG136 first, or a mixture of the two isolates.

Kernel washing. Kernels (5 g) were washed twice (gently hand shaken for 1 min) in 15 ml of 1% Tween 80, water, hexane, or ethanol. Kernels were air dried overnight and then surface disinfected as described above. After hot-water disinfection, kernels from each washing treatment were inoculated with YV04 1 h prior to inoculation with CG136. As a control treatment, unwashed kernels were inoculated with YV04 followed by CG136 as in the sequence of the inoculation experiment. An additional unwashed kernel treatment utilized the same isolate order but with conidia suspended in 1% Tween 80.

DNA isolations. Kernels and conidia were analyzed separately so the proportion of total *A. flavus* DNA comprised of each isolate from both kernel-infecting hyphae and conidia on the kernel surface could be determined. Conidia were washed from surfaces by agitating (for 10 s) kernels in 20 ml 0.1% Tween 80 followed by 20 ml water and collected by filtration through Miracloth. Maize kernels were dried (60°C, 48 h) and then pulverized for 10 s in an analytical mill (IKA Works, Wilmington, NC). DNA was isolated separately from conidia and 200 mg kernels using the FastDNA Spin kit and the FastPrep instrument (MP Biomedicals, Santa Ana, CA).

PCR and pyrosequencing. Pyrosequencing assays were developed previously based on single nucleotide polymorphisms (SNPs) that distinguish CG136 from other VCGs (30). The primer pair CG136-Afl-F/CG136-Afl-R was used to distinguish CG136 from the other isolates; CG136-Cmd-F/CG136-Cmd-R was used to distinguish YV04 from MR17 and EB01. The primers CG136-Afl-F and CG136-Cmd-R were 5' biotinylated and high-performance liquid chromatography (HPLC) purified. PCR conditions were described previously (30).

Amplicons were prepared for pyrosequencing analysis using a vacuum prep tool (Qiagen, Valencia, CA) as described previously (13, 30) and according to the manufacturer's instructions. Briefly, amplicons (40 µl) were immobilized on streptavidin-coated beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH

7.6) by shaking at room temperature for 10 min. Bead-bound amplicons were captured on the filter probes of the vacuum prep tool (Qiagen), washed with 70% ethanol, denatured with 0.2 M NaOH, washed with buffer (10 mM Tris-acetate, pH 7.6), and released into wells of a PSQ 96 sequencing plate (Qiagen) containing annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate, pH 7.6) and 0.5 μ M sequencing primer (CG136-Afl-S2 or CG136-Cmd-S). The sequencing plate was incubated at 90°C for 10 min and then cooled to anneal the primer to the single-stranded DNA.

Pyrosequencing was performed using a PSQ SNP reagent kit and a PSQ 96MA pyrosequencer according to the manufacturer's instructions (Qiagen). As deoxynucleoside triphosphates (dNTPs) are added to the reaction mix, the pyrosequencing reaction produces quantities of light proportional to quantities of each nucleotide incorporated into the single-stranded template. Relative quantities (percentages) of each nucleotide incorporated at the isolate-specific SNP were calculated using the allele quantification option of the PSQ 96MA 2.1 software program (Qiagen). Percentages of isolate-specific SNPs represent the proportions of total *A. flavus* DNA from kernel-infecting hyphae and conidia comprised of DNA from the isolate identified by that SNP.

Data analysis. Percentage data were arcsine transformed prior to analysis. The effects of inoculum mixture and sequence of inoculation on percentages of individual isolates in kernels and conidia were tested with a factorial analysis of variance (ANOVA). Percentages of the two coinfecting isolates were compared using a paired *t* test. Increases in competitive ability due to the inoculation sequence were calculated by subtracting the percentage of an isolate in mixed inoculum treatments from the percentage of an isolate in kernels or conidia when that isolate was inoculated 1 h prior to a second isolate. The increase in competitive ability conferred by prior host contact was compared among inoculation mixtures with an analysis of variance and was compared between isolates within mixtures using Student's *t* test. Correlations between the inoculum concentration and the percentage of the first isolate inoculated were performed. Effects of conidial binding and kernel-washing treatments on isolate percentages were each tested with an analysis of variance. For experiments without a trial by treatment interaction, data from the two trials were combined. When treatment effects were significant, means were separated with Fisher's least-significant-difference (LSD) test. Statistical analyses were performed using the SAS 9.1 software program (SAS Institute, Cary, NC).

RESULTS

Sequence of inoculation. Competition between isolates during kernel infection was influenced by the sequence of inoculation, with the first isolate to contact the host acquiring a competitive advantage during both kernel invasion ($P < 0.0001$) and sporulation on kernel surfaces ($P < 0.0001$) (Table 1). The competitive success of an individual isolate was also influenced by the identity of the coinfecting isolate (kernels, $P < 0.0001$; conidia, $P < 0.0001$), but being first to contact the host increased the competitive advantage regardless of which isolates were coinoculated. There was no interaction between the sequence of inoculation and coinoculation treatments (kernels, $P = 0.77$; conidia, $P = 0.74$) or between these treatments and the trial (kernels, $P = 0.43$; conidia, $P = 0.57$). Inoculation of kernels with mixed suspensions of conidia revealed competitive differences among isolates. YV04 and CG136 were competitively similar, with each contributing approximately 50% of the *A. flavus* DNA from either conidia produced on infected kernel surfaces or kernel-invading hyphae. MR17 was significantly less competitive than either YV04 or CG136 (Table 1) during both kernel invasion and sporulation. Although MR17 was the least competitive of the isolates examined, competitiveness improved when the isolate was introduced to kernels prior to one of the other isolates. The proportion of DNA from kernel-invading *A. flavus* hyphae attributable to MR17 increased from 39 to 50% when CG136 was inoculated second rather than simultaneously with MR17, and it increased from 25 to 38% when YV04 was inoculated second rather than

TABLE 2. Magnitude of competitive advantage conferred to the first *A. flavus* isolate to contact maize kernel surfaces

Isolates used (A, B)	Increase in % <i>A. flavus</i> DNA contributed by indicated isolate (SE) ^a					
	Kernels			Conidia		
	Isolate A first ^b	Isolate B first ^c	<i>P</i> value ^d	Isolate A first	Isolate B first	<i>P</i> value ^d
CG136, YV04	16 (6)	20 (6)	0.67	24 (5)	19 (4)	0.43
CG136, MR17	20 (5)	11 (8)	0.34	18 (2)	22 (4)	0.33
YV04, MR17	11 (7)	13 (7)	0.88	24 (4)	14 (2)	0.03
<i>P</i> value ^e	0.58	0.63	...	0.39	0.28	...

^a For each replicate, the percentage of *A. flavus* DNA comprised of the indicated isolate when isolates were inoculated simultaneously was subtracted from the percentage of the isolate when that isolate was inoculated first.

^b Kernels were inoculated with isolate A 1 h prior to inoculation with isolate B.

^c Kernels were inoculated with isolate B 1 h prior to inoculation with isolate A.

^d Competitive advantage conferred to an isolate inoculated prior to versus simultaneously with another isolate was compared between isolates A and B with Student's *t* test ($n = 4$).

^e An analysis of variance was used to test for differences among isolate pairs in the competitive advantage conferred to the isolate inoculated first. No significant differences were detected.

simultaneously. The magnitude of the advantage conferred to the first isolate compared to results of mixed-inoculum treatments did not differ significantly among individual isolates or isolate pairs (Table 2). When averaged across all comparisons, the percentage of total *A. flavus* DNA contributed by an isolate increased $20\% \pm 2\%$ for conidia and $15\% \pm 2\%$ for kernel-invading hyphae when the isolate was inoculated 1 h prior to the second isolate compared to results of simultaneous inoculation.

Inoculum concentration. When conidia from isolate YV04 were applied to kernels 1 h prior to an equal quantity of CG136, the inoculum concentration had a significant effect on the advantage conferred to YV04 during both kernel invasion ($P < 0.0001$) and sporulation ($P < 0.0001$). The influence of the inoculum concentration on the advantage conferred by initial host contact differed among trials (kernels, $P = 0.008$; conidia, $P = 0.02$), although the trend was similar (Fig. 1). Since there was a trial by inoculum concentration interaction (kernels, $P = 0.0003$; conidia, $P = 0.02$), the two trials were analyzed separately. For both trials, there was a significant positive linear correlation between the log of the quantity of conidia applied and the extent to which the first isolate, YV04, displaced CG136 (Table 3 and Fig. 1). The first isolate did not gain a competitive advantage at the lower inoculum concentrations in either trial. Thus, when 10^2 conidia of each isolate were applied to the kernels, the sequence of inoculation had no influence and the two isolates were equally competitive during kernel invasion (paired *t* test, trial 1, $P = 0.26$; trial 2, $P = 0.83$) and sporulation (paired *t* test, trial 1, $P = 0.55$; trial 2, $P = 0.60$).

Binding inhibition assay. Coating kernels with dead conidia of isolate EB01 did not interfere with the competitive advantage conferred to isolate YV04 by initial host contact (Table 4). Similar results were obtained by coating kernels with conidia killed with either heat or ethanol and with conidial fragments generated by passage through a French cell. The competitive

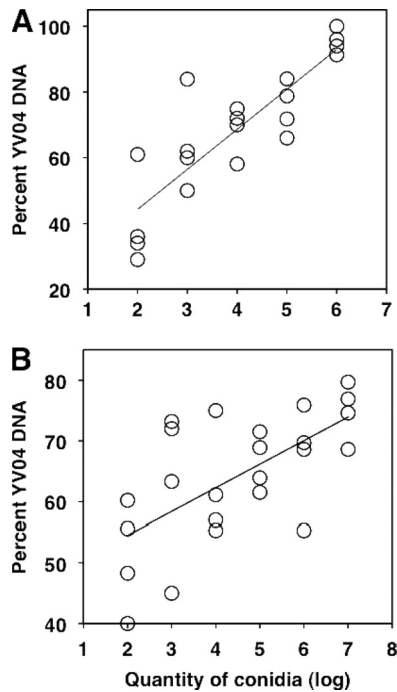


FIG. 1. Relationship between the inoculum concentration and the proportion of kernel-infecting hyphae attributable to isolate YV04. YV04 was inoculated 1 h prior to inoculation of an equal concentration of CG136 conidia. The log of the number of conidia inoculated per 5 g kernels is indicated along the x axis. Pearson correlation coefficients and *P* values are shown in Table 3. (A) Trial 1; (B) trial 2.

ability of YV04 in coinfection of kernels with CG136 was the same on Pioneer hybrid 33B50 and 33B54 when YV04 was inoculated first (33B50, 80% ± 3% of *A. flavus* DNA from kernels; 33B54, 78% ± 4%), second (33B50, 42% ± 8%; 33B54, 43% ± 4%), and when the isolates were mixed prior to inoculation (33B50, 65% ± 3%; 33B54, 65% ± 7%). DNA

TABLE 3. Relationship between inoculum concentration and proportion of total *A. flavus* DNA comprised of that from the first isolate to contact maize kernel surfaces

Trial	Sample type ^b	<i>r</i> value ^c	<i>P</i> value ^d	% YV04 (SE) ^a	
				Low inoculum ^e	High inoculum ^f
1	Kernel	0.86	<0.0001	40 (7)	96 (2)
	Conidia	0.78	<0.0001	43 (11)	98 (1)
2	Kernel	0.63	0.0009	51 (4)	75 (2)
	Conidia	0.57	0.004	55 (9)	80 (2)

^a *A. flavus* isolate YV04 was inoculated 1 h prior to inoculation of an equal concentration of CG136. The percentage of YV04 DNA is given.

^b *A. flavus* DNA from kernel-infecting hyphae and that from conidia from the kernel surface were analyzed separately.

^c Pearson correlation coefficient for the relationship between the log quantity of conidia from each isolate inoculated and the proportion of total *A. flavus* DNA comprised of isolate YV04. Ten-fold increases in inoculum from 10² to 10⁶ conidia per 5 g kernels were tested in the first trial, and 10² to 10⁷ conidia were tested in the second trial. For trial 1, *n* = 20; for trial 2, *n* = 24.

^d *P* value for Pearson correlation coefficients describing the relationship between log quantity of conidia and percent YV04 DNA.

^e Kernels were inoculated with 10² conidia of each isolate.

^f Kernels were inoculated with 10⁶ and 10⁷ conidia of each isolate in the first and second trials, respectively.

TABLE 4. Effect of coating maize kernels with dead conidia on competitive advantage conferred to the first *A. flavus* isolate inoculated

First isolate ^b	Type of dead conidia ^c	% YV04 DNA (SE) ^a	
		Kernels	Conidia
YV04	None	80 (1) a	73 (1) a
YV04	Fragments	82 (2) a	73 (4) a
YV04	Ethanol treated	78 (3) a	69 (3) a
YV04	Heat treated	78 (3) a	73 (3) a
Mix	None	67 (2) b	58 (4) b
CG136	None	52 (6) c	37 (4) c

^a Proportion of total *A. flavus* DNA comprised of that of YV04. *A. flavus* DNA from kernel-infecting hyphae and conidia from kernel surfaces were analyzed separately. Significant differences were detected among treatments with an analysis of variance (for kernels, *P* < 0.0001; for conidia, *P* < 0.0001). Means followed by the same letter are not significantly different by Fisher's LSD test.

^b The first isolate (YV04 or CG136) was inoculated 1 h prior to the second isolate. "Mix" indicates that conidia of the two isolates were mixed prior to inoculation and thus the two isolates were introduced to the host simultaneously.

^c Kernels were coated with conidia of isolate EB01 rendered nonviable by suspension in either 80°C water or 80% ethanol for 10 min. Fragments of conidia cell walls were produced using a French cell (see Methods).

from isolate EB01 was not detected by pyrosequencing, confirming that EB01 conidia were not viable.

Kernel washing. When maize kernels were inoculated with YV04 1 h prior to inoculation with CG136, proportions of isolate YV04 infecting kernels and sporulating on kernel surfaces differed among washing treatments (kernels, *P* = 0.03; conidia, *P* = 0.0002) (Table 5). Results from the two trials were significantly different (kernels, *P* = 0.0004; conidia, *P* < 0.0001), but there was no trial by treatment interaction (kernels, *P* = 0.50; conidia, *P* = 0.90). Kernels from the Tween 80 washing treatment had a greater proportion of YV04 conidia than kernels from other kernel-washing treatments and the unwashed control. The proportion of kernel-infecting hyphae comprised of YV04 in kernels washed with Tween 80 did not differ from that in kernels washed with water but was significantly different from the proportion of YV04 in unwashed kernels. For both kernel-infecting hyphae and sporulation, the

TABLE 5. Effect of maize kernel washing treatments on competitive advantage conferred to the first *A. flavus* isolate to contact maize kernels

Kernel wash ^b	Inoculum suspension ^c	% YV04 DNA (SE) ^a	
		Kernels	Conidia
Tween 80	Water	81 (3) a	78 (2) a
Water	Water	77 (2) ab	68 (3) b
None	Water	74 (3) b	69 (2) b
Hexane	Water	75 (2) b	67 (2) b
Ethanol	Water	70 (2) b	66 (3) b
None	Tween 80	72 (4) b	65 (3) b

^a Proportion of total *A. flavus* DNA comprised of that of YV04. Isolate YV04 was inoculated 1 h prior to inoculation of CG136. *A. flavus* DNA from kernel-infecting hyphae and conidia from kernel surfaces were analyzed separately. Means followed by the same letter are not significantly different by Fisher's LSD test.

^b Kernels were submerged in each of the designated solutions and washed by gentle agitation. "None" indicates kernels were not subjected to a washing treatment prior to inoculation.

^c Conidial inoculum for each isolate was suspended in either water or 1% Tween 80.

first isolate advantage was the same for unwashed kernels and kernels washed either with hexane or with ethanol. Addition of Tween 80 to the conidial suspension used for the inoculum had no influence on the competitive advantage of the first isolate (Table 5).

DISCUSSION

In a previous study (30), single isolates from 38 VCGs, including YV04 and MR17, were inoculated 1 h prior to inoculation of a test isolate from VCG CG136 and evaluated for competitive ability. MR17 was the least competitive isolate, and the competitive ability of YV04 was equal to or greater than that of any other isolate examined. In the current study, the sequence of inoculation significantly influenced competitive ability, with the first isolate in contact with host surfaces acquiring an advantage. These results suggest that simultaneous exposure of isolates to host tissues provides the best opportunity to measure the innate competitive abilities of isolates. However, ranking of isolates for competitive ability against a test isolate is apparently not influenced by the inoculation sequence. For example, YV04 competed better against CG136 than did MR17 whether the isolates were added before, after, or simultaneously with CG136. Thus, ranking of isolates in the previous study would presumably be the same had the isolates been mixed with CG136 prior to inoculation. However, because CG136 was always inoculated second, this isolate's competitive ability was underestimated. The highly competitive isolate YV04 significantly outcompeted CG136 when inoculated 1 h prior, but when isolates were inoculated simultaneously in the current study, CG136 and YV04 were equally competitive. Therefore, CG136 is highly competitive and likely as competitive as or more competitive than the 38 isolates previously screened for differences in competitive ability (30).

The magnitude of the advantage conferred to the first isolate to reach the host was equal for all examined isolates and was independent of innate competitive ability. This suggests that competitive displacement of one *A. flavus* isolate by another is determined by two factors: (i) innate differences in competitive ability, which are observed when isolates are inoculated simultaneously, and (ii) the advantage conferred by prior access to the host, which is equivalent among isolates at any given inoculum concentration (i.e., a 15% increase at the inoculum concentration used in the sequence of inoculation experiment). Although mechanisms contributing to these two factors are unknown, data from this study suggest the second factor is gradient driven and the result of limiting resources (e.g., attachment sites or nutrients). When coinfecting isolates are applied in equal quantities, the advantage conferred to the first isolate increases with the inoculum concentration, and at low concentrations, prior access to the host no longer results in a competitive advantage for the first isolate. At low inoculum concentrations, an as yet unidentified resource is apparently in excess relative to the number of conidia, and competition for that resource does not contribute to competitive exclusion. At high inoculum concentrations, however, this putative resource is largely depleted by the initial isolate, leaving subsequent isolates at a disadvantage.

This is the first study to identify an influence of the inocu-

lation sequence with only a single hour between inoculations of coinfecting *A. flavus* isolates. Previous studies have suggested the inoculation sequence is important for the efficacy of biological control by atoxigenic *A. flavus* (6, 8, 11) and other biocontrol agents (28, 42) but on time scales (24 to 96 h) adequate for spore germination and capture of physical (i.e., colonization of host surfaces) and nutritional resources. These prior studies suggested that establishment of the biocontrol isolate is necessary prior to challenge for enhancement of competitive displacement (11, 28, 42). The ability of initial host contact to confer an immediate competitive advantage suggests a stage in host colonization and infection not detected by previous studies. The exact nature of this new stage is not clear. Since a competitive advantage was acquired upon initial host contact and prior to germ tube emergence and host colonization, we hypothesized the advantage was mediated by competition among isolates for limited attachment sites on host surfaces. Specific mechanisms involved in attachment of *A. flavus* to host surfaces have not been characterized, but the immediate effect of the inoculation sequence in the current study indicated passive binding is most likely (41). Attempts to interfere with attachment by blocking binding sites with dead conidia (36), modifying the kernel surface by washing with solvents to remove pericarp wax (19, 37), and inhibiting hydrophobic interactions (16) by suspending the conidial inoculum in Tween 80 did not alter the competitive advantage conferred to the isolate inoculated first. Washing kernels with Tween 80 prior to inoculation increased the first isolate advantage, but the magnitude of this advantage was smaller than that resulting from the inoculation sequence. These results suggest that competition for attachment sites on the kernel surface does not account for the first-isolate advantage. Novel approaches may be necessary to determine mechanisms mediating this phenomenon.

The great precision and reproducibility of SNP quantification with pyrosequencing (13, 30) allowed for consistent resolution of statistically significant differences in competitive advantage in the current study. Colonization processes during intraspecific competition have been visualized with fluorescently labeled fungi (5), but such methods are qualitative or semiquantitative at best. For determining proportions of competing microorganisms, pyrosequencing requires only a single SNP to distinguish between closely related isolates or species, and the higher level of sensitivity and precision of pyrosequencing than of real-time PCR allows for greater consistency and more frequent statistical separation of treatment means (1, 30). Few studies in either microbial ecology or plant pathology have employed pyrosequencing to quantify individual isolates during isolate-isolate interactions on hosts and/or in the environment. The great precision in both the current and previous studies (13, 30) demonstrates pyrosequencing to be a powerful tool for the quantification of competitive interactions among microorganisms.

Though the current study did not strive to simulate *A. flavus* population dynamics in the field, data suggest previously unknown intraspecific interactions influence compositions of *A. flavus* communities. Mixtures of isolates, VCGs, and strains are resident in soils and air and on crops (2, 4, 22, 33, 38), so competitive interactions resulting from simultaneous exposure of crops to multiple isolates may drive *A. flavus* population

dynamics in the field. Isolates with the greatest innate competitive and reproductive ability will be the most successful during crop invasion and epidemic increases in *A. flavus* populations. However, the compositions and densities of crop-associated *A. flavus* populations naturally vary during crop development (4, 33). In the current study, isolate MR17 was outcompeted by both YV04 and CG136 when isolates were coinoculated but not when MR17 reached the host first. These results suggest the first isolate to contact the host during susceptible stages in crop development will have a competitive edge regardless of innate competitive ability; this is one mechanism by which relatively poor competitors may be maintained in *A. flavus* populations rather than being displaced by highly competitive isolates over time.

Crops are exposed to highly variable inoculum concentrations that may range from lower than to higher than the doses examined in the current study (4, 33). In the field, the conidial inoculum is brought to maize kernels directly by insects, or the fungus colonizes silks and grows down to the kernels (34). It is unknown if the order of host exposure has the same influence on colonization and infection of tissues other than kernels (i.e., silks) or damaged tissues (i.e., insect-damaged kernels). Results of the current study may be most applicable to routes of infection that involve direct contact of kernels with conidial inoculum, such as exposure to *A. flavus* during harvest and handling prior to storage. Regardless, the phenomenon quantified in the current study should be taken into consideration when designing competition experiments for *A. flavus* and, potentially, other microorganisms. The ability to rapidly "capture" substrates prior to colonization may be an important component of microbial competition in general.

Previously, the ability to sporulate during competition was not equivalent to competitive ability during host tissue ramification for all isolates examined (30). Sporulation during competition is an important characteristic of potential biocontrol isolates since the capacity to sporulate on infected hosts contributes to isolate success during epidemics. The current study suggests additional criteria for selection of premier biocontrol isolates. Superior competitive ability is important, but prior host contact by aflatoxin producers may reduce the ability of highly competitive biocontrol isolates to reduce aflatoxin contamination through competitive exclusion. Traits of atoxigenics favoring early dispersal to the host, therefore, are as important as the innate ability to compete during host tissue ramification. Current use of atoxigenic strains for aflatoxin management seeks to distribute the biocontrol agent prior to colonization of susceptible crop components by aflatoxin producers and typically results in over 80% displacement of the resident *A. flavus* community (12); this is consistent with the current results, which suggest atoxigenics will be most effective when making host contact prior to aflatoxin producers. Variation in the composition of *A. flavus* communities over time suggests isolates are adapted to different seasons or environmental conditions (4, 33). Thus, both proper timing of application and selection of atoxigenics that sporulate and disperse under conditions and timing coincident with susceptible stages in crop host development will improve the efficacy of biocontrol by atoxigenic *A. flavus*.

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