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Mutual Exclusion between Fungal Species of the *Fusarium* Head Blight Complex in a Wheat Spike

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***Fusarium* head blight (FHB) is one of the most damaging diseases of wheat. FHB is caused by a species complex that includes two genera of Ascomycetes: *Microdochium* and *Fusarium. Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, and *Microdochium nivale* are among the most common FHB species in Europe and were chosen for these experiments. Field studies and surveys show that two or more species often coexist within the same field or grain sample. In this study, we investigated the competitiveness of isolates of different species against isolates of *F. graminearum* at the scale of a single spike. By performing point inoculations of a single floret, we ensured that each species was able to establish independent infections and competed for spike colonization only. The fungal colonization was assessed in each spike by quantitative PCR. After establishing that the spike colonization was mainly downwards, we compared the relative colonization of each species in coinoculations. Classical analysis of variance suggested a competitive interaction but remained partly inconclusive because of a large between-spike variance. Further data exploration revealed a clear exclusion of one of the competing species and the complete absence of coexistence at the spike level.**

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Fusarium head blight (FHB) is a very damaging wheat disease that occurs in many wheat-growing areas worldwide. It is caused by a species complex, the composition of which varies with geography and time (1, 2). The main species present in Europe are *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium poae*, *Microdochium majus*, and *Microdochium nivale* (3, 4). *F. graminearum* is usually the predominant species in population surveys, but several FHB-causing species are commonly found in the same fields, especially when the climatic conditions are favorable to the disease (2, 5, 6). However, the way that these species interact on the host plant remains poorly understood.

Most studies on the direct interaction between *Fusarium* species have been done on laboratory media, but *in vitro* interaction studies are of limited use in predicting the outcome of fungal species competition *in planta* (7, 8). In the few available *in vivo* studies, interactions among *Fusarium* species have been identified as competitive in most cases. Simpson et al. (7) studied interspecific interactions between *F. culmorum* and the two *Microdochium* species on wheat seedlings and *in vitro*. They showed that *F. culmorum* was a better competitor and inhibited colonization by *Microdochium* spp. When *M. majus* was well established in the host, however, it was able to suppress *F. culmorum*. In an analysis of interactions among isolates of four FHB species, Xu et al. (9) found that the interaction was competitive in most cases, leading to a reduced fungal biomass in coinoculations compared to single-isolate inoculations. They measured an increase in mycotoxin production in the coinoculations, contrary to the results obtained by Siou et al. (10). A general trend in these studies is that the most competitive species does not colonize the host in coinoculations significantly more extensively than when inoculated alone (i.e., no advantage is provided by the presence of the other species), but the species with the selective disadvantage develops less extensively in the presence of a competitor than when infecting alone (2). This was once again observed by Siou et al. (10, 11). Such competitive interactions seem to occur at the intraspecific level as well. Miedaner et al. (12) investigated interactions among four strains of *F. culmorum* on rye in field epidemics and found that coinoculations of several isolates in the same plot led to a reduced disease severity.

Interactions among *Fusarium* species can nevertheless be synergistic in certain situations. In a field experiment on maize, Reid et al. (13) found that the total fungal biomass was higher in ears inoculated with both *F. graminearum* and *F. verticillioides* than in ears inoculated with *F. verticillioides* alone. Using the same pathosystem, Picot et al. (14) inoculated maize ears either with a spore mixture of both fungal species or using a sequential procedure consisting of a first inoculation with *F. graminearum* followed by a second with *F. verticillioides* 1 week later. They found that previous contamination by *F. graminearum* can facilitate subsequent infections of maize ears by *F. verticillioides*. Positive interactions among FHB species are sometimes suggested by population surveys. For example, Xu et al. (15) performed a population survey of the FHB species on wheat in four European countries and observed that the frequencies of several species were positively correlated. However, they noted that this may be the result of similar responses to climatic conditions rather than of synergistic effects.

The studies on FHB species competition reviewed above were based on global inoculations of the wheat spikes, usually by spraying a spore suspension on the whole spike. In the case of field

Received 13 February 2015 Accepted 27 April 2015
Accepted manuscript posted online 1 May 2015
Citation Siou D, Gélisse S, Laval V, Suffert F, Lannou C. 2015. Mutual exclusion between fungal species of the *Fusarium* head blight complex in a wheat spike. Appl Environ Microbiol 81:4682–4689. doi:10.1128/AEM.00525-15.
Editor: D. Cullen
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00525-15.
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doi:10.1128/AEM.00525-15

TABLE 1 Isolates used in the experiments

Species[a]	Isolate	Chemotype[b]	Aggressiveness[c]	Use in expt in[d]:	
				2010	2011
F. graminearum	fg91	NIV	++	-	×
	fg159	DON	+	-	×
	fg165	DON	++	×	-
	fg178	DON	+++	×	×
F. culmorum	fc124	DON	+++	-	×
	fc233	DON	+++	-	×
	fc129	NIV	++	×	×
	fc337	NIV	+++	×	×
F. poae	fp3	NIV	+	-	×
	fp6	NIV	+	-	×
M. nivale	mn224		−	-	×
	mn227		−	-	×

[a] *F. graminearum* was used in challenges with isolates of other species. For each pair of isolates, two treatments were performed with *F. graminearum* as the challenger (inoculated above the other isolate) or as the competitor (inoculated below). In addition, each isolate was inoculated alone in order to evaluate its aggressiveness level.
[b] Main toxin produced. NIV, nivalenol; DON, deoxynivalenol.
[c] Average aggressiveness of the *Fusarium* isolates as measured on wheat spikes in a greenhouse in previous experiments. −, +, ++ and +++ indicate very low, low, moderate, and high aggressiveness, respectively.
[d] ×, used; -, not used.

epidemics, however, the disease is likely to result from single-spore infections or at most from a few successful infections per spike. Zeller et al. (16) estimate that *F. graminearum* produces between two and three successful infections per spike during an epidemic in a wheat field, even though more spores are deposited. Under such conditions, the output of the competition between two isolates results both from their capacity for host infection and from their capacity for spike colonization. A limitation of artificial spray inoculations is that they do not allow the expression of differences in the colonization capacity of the strains (8, 17), which is clearly an important aggressiveness component of the *Fusarium* species (18, 19). It is generally accepted that single-spikelet (point) inoculation makes it possible to assess differences in the pathogens' ability to spread within the spike, whereas spraying a conidial suspension on spikes assesses the combined effect of infection efficacy and colonization capacity (12, 20).

To further increase our understanding of FHB species interactions, we studied isolate competition for spike colonization after single floret inoculations. With this approach, the local establishment of each isolate was independent (i.e., there was no competition for initial infection) and the isolates were competing only for spike colonization. We investigated how the concomitant presence of *F. graminearum* and another of the FHB species influenced their relative colonization capacity. For that, we compared the colonization of *F. graminearum* strains alone or in competition with *F. culmorum*, *F. poae*, and *M. nivale* after local inoculations of individual wheat spikes. The fungal biomass of each species in each spike was evaluated by quantitative PCR.

MATERIALS AND METHODS

Plant material. A greenhouse experiment was carried out in 2010 and replicated in 2011. Seeds of winter wheat, cv. Royssac, considered to be highly susceptible to FHB, were sown in Jiffy peat pots and kept for 2 weeks under greenhouse conditions for seedling emergence. Seedlings were vernalized in a growth chamber for 8 weeks at 8°C with a 10-h light period and a 14-h dark period. They were then individually transplanted into pots containing 1 liter of commercial compost (Klasmann peat substrate 4; Klasmann France SARL, France), with 2 g of slow-release fertilizer (Osmocote Exact 16-11-11 N-P-K 3MgO Te). Pots were placed in a greenhouse compartment at 15 to 20°C with a 15-h day photoperiod. During plant growth, natural daylight was supplemented with 400-W sodium lights between 6:00 a.m. and 9:00 p.m. Plants were fertilized with Hydrokani C2 (Hydro Agri Spécialités, France) at a 1:100 dilution rate. The flowering date of each spike was recorded at the beginning of anthesis. The plants were sprayed with metrafenone (Flexity; 1 ml liter^{-1}; Bayer CropScience, Germany) and lambda-cyhalothrin (0.2 ml liter^{-1}; Karaté Zéon; Syngenta Agro S.A.S., France) to control powdery mildew (*Blumeria graminis*) and insects, respectively, during growth. These products were tested before the experiment and showed no effect on FHB.

Fungal material. The fungal isolates used in the experiment are described in Table 1. The *F. graminearum* and the *F. culmorum* isolates were provided by F. Forget (INRA Bordeaux, France), the *F. poae* isolates were provided by Bayer CropScience, and the *Microdochium* isolates were provided by A.-S. Walker (INRA, Versailles-Grignon, France). They all originated from field samplings in France. For spore production, the isolates were grown in petri dishes on potato dextrose agar (PDA; 39 g liter^{-1}) at 19°C and exposed to light for 3 days. For *Fusarium*, four mycelial plugs were then transferred to 250 ml of carboxymethyl cellulose (CMC) broth (7.5 g of CMC, 0.5 g of yeast extract, 0.5 g of $MgSO_4$, 0.5 g of NH_4NO_3, and 0.5 g of KH_2PO_4 per liter) with continual shaking. After 3 days, the medium was filtered through cheesecloth to collect the spores. For *Microdochium*, the isolates were transplanted twice in petri dishes with PDA, maintained at 19°C, and exposed to light for 5 days. Five milliliters of sterile water was then added to each petri dish to collect the spores. For each isolate, a spore suspension in sterile distilled water was adjusted to a concentration of 2×10^4 conidia ml^{-1} using a Malassez cell. The suspensions were then stored at 4°C until they were used for inoculation, which took place on the same day. After inoculation, a few microliters of each spore suspension was deposited on PDA to check spore viability.

Experimental design and inoculation procedure. This experiment was conducted twice, in September-October 2010 and in April-May 2011, with the same cultivar and using the same protocols for inoculation and disease assessment. *F graminearum* isolates were inoculated in competition with one of the other isolates (*F. culmorum* isolates only in 2010 and

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TABLE 2 Primer and probe sequences and qPCR amplification conditions for each species

Target species	Primer or probe	Sequence	Reporter/quencher (5′/3′)	Final concn (nM)	Annealing temp (°C)
F. graminearum	EF1-FCFG_F	TCGATACGCGCCTGTTACC		300 nM	62
	EF1-FG_R	ATGAGCGCCCAGGGAATG		300	
	grami2-EF1_rev	AGCCCCACCGGGAAAAAAATTACGACA	FAM/TAMRA	100	
F. culmorum	EF1-FC_F2	CGAATCGCCCTCACACG		300	62
	EF1-FC-R2	GTGATGGTGCGCGCCTAG		300	
	culmo2-EF1-R2	ATGAGCCCCACCAGAAAAATTACGACAA	FAM/TAMRA	100	
F. poae	EF1-FP2_F	CTCGAGCGATTGCATTTCTTT		300	60
	EF1_FP2_R	GGCTTCCTATTGACAGGTGGTT		300	
	EF1-FP	CGCGAATCGTCACGTGTCAATCAGTT	FAM/TAMRA	100	
M. nivale	Mniv_Btub_F	TCTACTTCAACGAGGTATGTCACCAT		300	62
	Mniv_Btub_R	CCTAAGTTATGTGGGTGGTCAGTTAG		300	
	Mniv_Btub	TTCGGGCTTCACACATTCGGCC	FAM/TAMRA	150	

each isolate of *F. culmorum*, *F. poae*, and *M. nivale* in 2011). The isolates used in each trial are indicated in Table 1. In addition, each of these isolates was inoculated alone in order to evaluate its aggressiveness level. For each treatment (i.e., isolate or isolate combination), two pots with four spikes each (2010; eight replicates) or five pots with two spikes each (2011; 10 replicates) were inoculated. The pots were randomized in the greenhouse. No tiller effect was detected within the same pot in previous experiments. This was tested again in this experiment, and since no effect was found, all the spikes were considered independent replicates.

In these trials, we compared colonization by a challenger isolate inoculated in the upper part of a spike in the presence and absence of a competitor in the lower part. *F. graminearum* was either the challenger or the competitor relative to isolates of the other species (Table 1). Each spike was inoculated at anthesis. A floret located in the middle of the spike was inoculated with the competitor isolate, and the challenger isolate was inoculated three spikelets above. In single inoculations, the isolates were inoculated at the same position along the spike as the challenger isolate in mixed inoculations. Five microliters of the spore suspension was deposited inside each inoculated floret with a micropipette. After inoculation, the spikes were enclosed in a sealed transparent polyethylene bag for 3 days to maintain 100% relative humidity and to promote infection (11, 21).

Assessment of spike colonization. The spikes were observed from 5 days after inoculation (appearance of first symptoms) to 25 days after inoculation. The number of spikelets with FHB symptoms was visually assessed three times a week. The percentage of diseased spikelets on each spike was calculated, and the appearance dynamics of symptomatic spikelets on each spike was characterized by the area under the disease progress curve (AUDPC) (see Data Set S1 in the supplemental material). The AUDPC was corrected by the number of days of observation, which differed slightly among the spikes.

The extent of spike colonization by each fungus was assessed by quantitative PCR, the amount of fungal DNA being commonly considered proportional to the fungal biomass (22). The use of fungal amplicons as a proxy for the biomass of the FHB species was supported by results of previous experiments (11). For each sample (i.e., each spike), the amount of fungal DNA was expressed relative to the amount of plant DNA in the same sample (10).

The total DNA was extracted from around 50 mg of grounded material using the DNeasy plant mini kit according to the manufacturer's instructions (Qiagen Ltd., Courtaboeuf, France). The DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Cambridge, United Kingdom) and diluted to a final concentration of 20 ng μl^{-1}. The amount of both fungal and wheat DNA was estimated by quantitative PCR (qPCR) for each inoculated spike. Species-specific primer pairs and species-specific TaqMan probes were used to enhance the specificity of the test (Table 2). The primers and probes were purchased from Eurogentec (Seraing, Belgium). All the probes used were TaqMan labeled with FAM (6-carboxyfluorescein)/TAMRA (6-carboxytetramethylrhodamine) quencher. Real-time PCR was carried out in a total of 25 μl consisting of 6.25 μl qPCR MasterMix with ROX (6-carboxy-X-rhodamine) and uracil *N*-glycosylase (UNG) at a final concentration of 1× (Eurogentec, Angers, France), species-specific primer and probe (Table 2), and 5 μl of template DNA. The samples were standardized based on the plant DNA quantified with plant EF1α real-time PCR primers and SYBR green technology as described by Nicolaisen et al. (22). For wheat DNA quantification, we used MESA green qPCR MasterMix Plus for SYBR assay (Eurogentec) at a final concentration of 1×, with the same primer concentration and 5 μl of template DNA diluted 1:10. The PCRs were performed in duplicate (and in triplicate on samples used for the standard curve) on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) in Applied Biosystems 96-well plates. The amplification conditions included an initial step of 2 min at 50°C, 95°C for 10 min, and 40 cycles of 15 s at 95°C and 60 s at 62°C (60°C for *F. poae*). DNA quantifications were done using standard curves of DNA from *F. graminearum*, *F. culmorum*, *F. poae*, or wheat extracted from pure cultures and noncontaminated wheat kernels. Each standard curve was generated by 10-fold dilution series ranging from 1.0 to 10^{-3} ng μl^{-1} for fungal DNA and 5.0 to 5.10^{-3} ng μl^{-1} for wheat DNA. Results were analyzed with AB SDS2.2.2 software (Applied Biosystems). PCR efficiency was on average 98%. The amount of fungal DNA was calculated from cycle threshold (C_T) values using the standard curve, and these values were normalized to the estimated amount of plant DNA based on the plant EF1α assay (22). In a preliminary methodological study, we established that the measured DNA amounts were representative of the species frequencies and were not biased by competition between PCR primers, templates in the test tube, or artifacts (unpublished data). Such verification was performed as stated by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines using nine FHB species (*F. graminearum*, *F. culmorum*, *F. poae*, *M. nivale*, *Fusarium sporotrichioides*, *Fusarium langsethiae*, *F. avenaceum*, *Fusarium tricinctum*, and *M. majus*). The specificity of the primers and probes was carefully checked *in vitro* using at least three isolates belonging to the four species involved in the current study.

In 2010, the spikes were harvested at maturity (55 days after inoculation) and divided into three parts: the lower part, including the spikelet inoculated with the competitor isolate (or not inoculated in single inoculations); the middle part, containing the two spikelets between the two inoculation points; and the upper part, containing the spikelet inoculated with the challenger isolate. Since it was apparent that the fungal growth

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TABLE 3 Experiment and isolate effects on disease severity (AUDPC) in single inoculations[a]

Source of variance	df	Mean square	*F* value	*P*
Isolate (A)[b]	11	0.637	25.41	<0.001
Expt (B)[c]	1	0.264	10.55	0.001
A × B	2	0.0653	2.61	0.078
Residuals	131	0.025		

[a] The isolates are indicated in Table 1.
[b] Isolate inoculated alone in the upper part of the spikes.
[c] Experiments in 2011 and 2012.

TABLE 4 Effect of the challenger and the competitor on the fungal biomass of the challenger[a]

Expt and source of variance	df	Mean square	*F* value	*P*
2010				
Challenger (A)	3	0.003	2.10	0.107
Competitor (B)	4	0.005	3.57	0.010
A × B	4	0.003	2.03	0.098
Residuals	83	0.001		
2011				
Challenger (A)	10	0.020	21.60	<0.001
Competitor (B)	11	0.005	5.55	<0.001
A × B	37	0.002	1.98	<0.001
Residuals	411	0.001		

[a] In this analysis, the competitor effect includes the single inoculations (absence of the competitor). The challenger was the isolate inoculated in the upper part of the spikes; the competitor was the isolate inoculated in the lower part of the spikes.

was mainly downwards along the spike (see Results) (17) and that competition between isolates occurred in the middle and lower parts of the spike, the procedure was simplified in 2011. In this trial, the part of the spike above the inoculation point of the challenger isolate was discarded and the rest was analyzed as a whole. To compare the two trials, we added the amounts of fungal DNA measured by quantitative PCR in the different parts of the spikes in 2010. In all cases, we took care to express the fungal DNA relative to the plant DNA for each spike. In 2010, the kernels were separated from the chaff and ground with a mixer mill (MM 400; Retsch, France) before DNA quantification. In 2011, the kernels and chaff were ground and analyzed together.

Statistical analyses. Analyses of variance (ANOVA) were used to identify potential sources of variability (experiment, species, isolate, and presence/absence of a competitor) that may influence the growth of an isolate. In 2010, the difference in fungal DNA content between the three parts of the spikes was tested by ANOVA. Multiple mean comparisons were done with Tukey's honestly significant difference (HSD) tests. Residual distribution was checked for linearity and variance homogeneity.

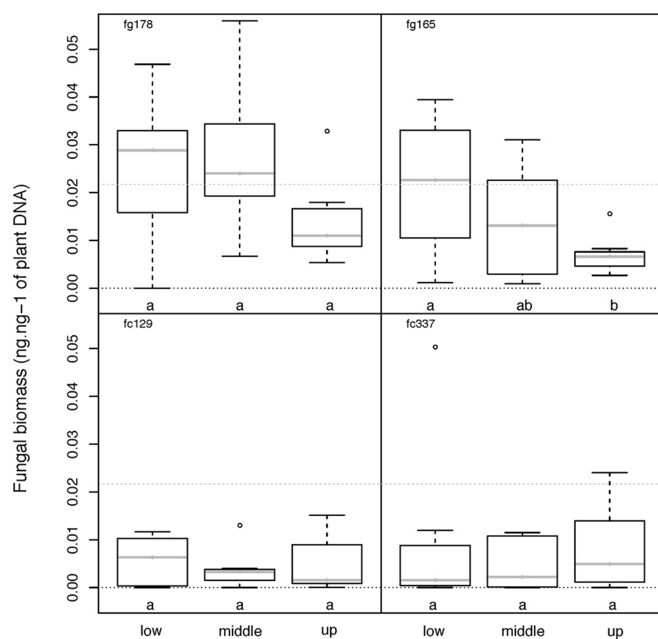

FIG 1 Fungal biomass quantified in the lower, middle, and upper sections of wheat spikes inoculated with a single *F. graminearum* isolate (fg178 or fg165) or an *F. culmorum* isolate (fc129 or fc337) in the 2010 experiment performed to evaluate aggressiveness levels in the absence of competition between two species. Each isolate was inoculated in a single floret of the upper section in order. The thick line within each boxplot shows the median value; the dashed line across each graph shows the overall mean value; the small open circle represents an outlier. Letters indicate significant differences among mean values for each isolate (Tukey's HSD test, $P = 0.05$). For fg178, the fungal biomass in the lower section was found to be significantly different when the outlier (open circle) was removed.

RESULTS

Visual assessment of disease severity. We first compared the visual assessment of disease development, as indicated by the AUDPC (area under the disease progress curve), of each isolate when inoculated alone on a spike. All isolates produced visible symptoms, with AUDPC values significantly different from zero (Student's test, $P < 0.05$), except for fp3, mn224, and mn227, which generally remained restricted to the inoculated spikelet. The analysis of variance (Table 3) showed a significant effect of the isolate, as indicated by lower AUDPC values for *F. poae* and *M. nivale* isolates than for *F. graminearum* and *F. culmorum* isolates.

Spike colonization by the fungus. Data from the single inoculations of the 2010 experiment were used to explore the dynamics of spike colonization by *F. graminearum* and *F. culmorum*. For each isolate, we compared the amount of fungal DNA quantified in the upper, middle, and lower parts of the spike. A first result is that fungal DNA was found for each isolate in all three parts of the spike, with values significantly above zero (Fig. 1). Either there was no significant difference between the three spike sections, or the lower part contained more fungal biomass. Both *F. graminearum* isolates developed more in the lower part, indicating a clear tendency to grow downward along the spike. This was less clear for the *F. culmorum* isolates, for which the fungal biomass was not found to be significantly different in the three spike sections. In all cases, this means that these species were, to some extent, able to colonize the whole spike from a localized infection.

Effect of the presence of a competitor on spike colonization. We tested the effect of the presence of a competitor isolate when a challenger isolate was inoculated above in the same spike. In the first variance analysis (Table 4), we evaluated the effects both of the challenger and of the competitor within each experiment. In these analyses, the "competitor" factor included the single inoculations, i.e., a level for which the competitor was actually absent. In 2010, the competitor effect was found to be significant, but the multiple mean comparisons did not make it possible to separate the different treatments. In 2011, both main effects and their interaction were significant, indicating that the output of the com-

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TABLE 5 Mean values for the fungal DNA quantified in wheat spikes inoculated with different isolates of *Fusarium* and *Microdochium* in 2011[a]

DNA measured	*F. graminearum* isolate	Level of DNA (pg ng^{-1} of plant DNA)[b]								
		F. culmorum				*F. poae*		*M. nivale*		
		fc124	fc129	fc233	fc337	fp3	fp6	mn224	mn227	SI
F. graminearum as challenger	fg91	15 a	16 a	17 a	38 a	25 a	21 a	19 a	20 a	78 a
	fg159	5 a	3 a	3 a	1 a	1 a	2 a	1 a	3 a	2 a
	fg178	33 b	61 ab	20 b	59 ab	30 b	49 ab	72 ab	62 ab	114 a
F. graminearum as competitor	fg91	15 a	31 ab	60 ab	36 ab	37 ab	45 ab	36 ab	130 b	
	fg159	1 a	001 a	2 a	1 a	1 a	1 a	2 a	3 a	
	fg178	105 a	121 a	63 a	123 a	123 a	84 a	75 a	116 a	
Non-*F. graminearum* competitor	fg91	115 a	40 a	64 a	36 a	<1 a	<1 a	1 a	2 a	
	fg159	52 a	15 a	36 a	18 a	1 a	2 a	1 a	<1 a	
	fg178	79 a	8 a	20 a	13 a	<1 a	<1 a	<1 a	<1 a	
Non-*F. graminearum* challenger	fg91	22 a	12 a	21 ab	9 a	<1 a	<1 a	<1 a	<1 a	
	fg159	67 ab	7 a	20 ab	9 a	<1 a	<1 a	1 b	1 a	
	fg178	13 a	7 a	3 a	14 ab	<1 a	<1 a	<1 a	1 a	
	SI	94 b	13 a	56 b	31 b	1 a	<1 a	<1 a	<1 a	

[a] The challenger isolate was inoculated above the competitor. SI, single inoculation.

[b] Within the same row, values followed by different letters are statistically significantly different ($P = 0.05$) according to the Tukey HSD test. Within the same column, values followed by different letters are statistically significantly different ($P = 0.05$) according to the Tukey HSD test.

petition was dependent on both the challenger and the competitor (including its absence) and on their specific combination. The 2011 data set was then further explored by performing a separate analysis for each isolate, either as a competitor or as a challenger (Table 5). Despite apparent tendencies, it was difficult to draw general conclusions from these analyses. The presence of a competitor tended to reduce the colonization by the challenger isolate, but the difference in measured fungal DNA was significant in a few cases only. The colonization capacity of fg178 was significantly reduced in the presence of fc124, fc233, and fp3. The same effect was observed for fc124 against fg91 and fg178, for fc233 against fg178, and for fc337 against fg91 and fg159 (Table 5). Inversely, the presence of a challenger did not seem to have a significant effect on the colonization by the competitor (except for fg91, which developed better in the presence of mn227). This comparison of inoculated individuals by ANOVA was disappointing. We then used another approach, taking advantage of the fact that each spike was inoculated and assessed individually.

Within-spike competition between isolates. As often occurs with FHB, the variability in disease assessment was high. In the present case, the variance in the amount of DNA produced by an isolate in a given spike made the comparisons between treatments difficult. Then, instead of comparing the spikes for the frequency of each species, we plotted the amount of challenger DNA versus the amount of competitor DNA for each spike. This allowed us to remove the effect of the between-spike variance and to directly relate the respective biomasses of the challenger and the competitor in each individual spike. Figure 2 shows a clear exclusion between the challenger and the competitor: when one of the isolates was able to colonize the spike, the other was always found in a small amount (with a single exception, in 2010). In order to formally confirm the effect observed in Fig. 2, we tested the effect of the presence of a well-developed competitor by considering a threshold in its colonization level and by comparing the growth of the challenger isolate when the competitor was above or below this threshold. The threshold was set at 5% of the maximal value of the fungal biomass of the competitor. An analysis of variance (Table 6) indicates a strong effect of the threshold (accounting for 55% of the total variance), but a significant challenger-threshold interaction reveals that not all isolates were equally affected by the presence of a well-developed competitor.

In order to take large differences in aggressiveness among the isolates into account, we repeated the analysis without the weakly aggressive *F. poae* and *M. nivale* isolates. The main effects were similar to the previous ones, with a lower influence of the experiment, and only the challenger-threshold interaction remained significant (data not shown). We then performed the same analysis again (Table 6) without isolate fg159, which was characterized by low aggressiveness (Table 1), and keeping only the aggressive isolates. Only the challenger effect and the threshold effect then remained significant. In this analysis, the threshold effect accounted for 64% of the total variance. This clearly established that the presence of a well-developed competitor thwarted the colonization of the spike by a challenger isolate and indicated that not all isolates were equally good challengers.

It seems, however, that even weakly aggressive isolates may have an influence on the growth of a challenger. Figure 2c shows that the presence of *F. poae* and *M. nivale* in the lower part of the spike could affect the growth of *F. graminearum*, since no high concentrations (above 0.04 ng ng^{-1}) of *F. graminearum* DNA (except one) were found in the spikes in which either *F. poae* or *M. nivale* was present above 0.001 ng ng^{-1}. The number of spikes

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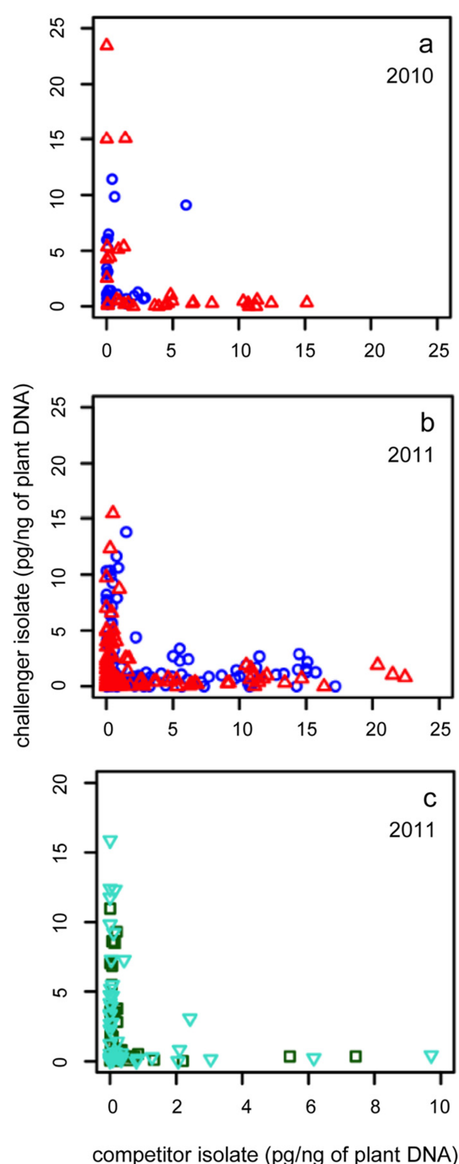

FIG 2 Fungal biomass of a challenger FHB isolate (inoculated in the upper part of a spike) expressed as a function of the fungal biomass of a competitor FHB isolate (inoculated in the lower part of a spike) in the 2010 (a) and 2011 (b and c) experiments performed to compare the colonization by a challenger in the presence or absence of a competitor. (a and b) Combinations of *F. graminearum* and *F. culmorum* isolates; (c) combinations of *F. graminearum* and *F. poae* or *M. nivale*. Blue circles, *F. graminearum* as challenger and *F. culmorum* as competitor; red triangles, *F. culmorum* as challenger and *F. graminearum* as competitor; green squares, *F. graminearum* as challenger and *F. poae* as competitor; turquoise inverted triangles, *F. graminearum* as challenger and *M. nivale* as competitor. Note that the *x* axis scale in panel c is different from that in panels a and b.

with a sufficient colonization by those competitors was too low for a formal analysis, but Fig. 2c indicates a clear separation of the data.

DISCUSSION

The main result of our study was that independent infections of a wheat spike by two isolates of different species led to a high degree of dominance by one of the isolates after the spike colonization. Our data clearly suggest that, when *F. graminearum* and *F. culmorum* infect the same spike, only one of the species will finally colonize the host, whereas the other will not develop or will develop to a very limited extent. Even when the competitor was a weakly aggressive isolate, its presence in the lower part of the spike still had an apparent effect on challenger colonization.

The point inoculation method, which consists of depositing a spore suspension in a single spikelet, is a technique often used for measuring the ability of the pathogen to colonize a wheat spike (17, 23) and has been successfully used to establish a difference in aggressiveness among *Fusarium* strains belonging to different subpopulations (8). In our study, the point inoculation method was used to distinguish the colonization capacity of the competing isolates from their infection efficiency, which cannot be done with spray inoculations. In combination with quantification by qPCR, it made it possible to investigate the competition for spike colonization after local infection by two different species.

Argyris et al. (17) describe two kinds of disease progression in the wheat spike: the local colonization of the fungus around the inoculation point (i.e., within the inoculated floret) and the colonization of the entire spike, with a marked tendency to progress toward the spike basis. Based on such observations, the host resistance has been characterized as type I, or resistance to initial infection, and type II, or resistance to spike colonization (24). In our experiment, we used a cultivar rated as susceptible to FHB and for which aggressive isolates were able to colonize the entire spike

TABLE 6 Analyses of variance to assess effects on the challenger fungal biomass of the challenger isolate, the competitor isolate, the threshold competitor colonization level, and the experiment

Source of variance[a]	df	Mean square	*F* value	*P*
With all isolates				
Challenger (A)	11	0.016	16.39	<0.001
Competitor (B)	12	0.004	4.29	<0.001
Threshold (C)	1	0.052	51.39	<0.001
Expt (D)	1	0.007	7.05	0.008
A × B	40	0.001	1.23	0.160
A × C	10	0.003	2.74	0.003
A × D	2	0.003	3.35	0.036
B × C	6	0.002	2.31	0.033
B × D	3	0.002	1.75	0.156
C × D	1	0.003	2.98	0.085
Residuals	477	0.001		
Without *F. poae* isolates, *M. nivale* isolates, and fg159				
Challenger (A)	6	0.009	5.02	<0.001
Competitor (B)	7	0.005	2.84	0.007
Threshold (C)	1	0.071	40.99	<0.001
Expt (D)	1	0.010	5.90	0.016
A × B	13	0.001	0.80	0.656
A × C	5	0.002	1.00	0.419
A × D	2	0.003	1.93	0.147
B × C	6	0.002	0.93	0.477
B × D	3	0.002	1.01	0.390
C × D	1	0.004	2.36	0.126
Residuals	224	0.002		

[a] The challenger is the isolate inoculated in the upper part of the spikes; the competitor is the isolate inoculated in the lower part of the spikes. "Threshold" is the factor characterizing the presence/absence of the competitor above a threshold. "Expt" refers to the 2010 or 2011 experiment.

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from an initial infection localized in a single floret. Schroeder and Christensen (24) suggested that the clogging of vascular tissue in the rachis above the initial infection causes the head to bleach and ripen prematurely, so that even kernels that are not directly infected are shriveled because of a shortage of water and nutrients. The difficulty of developing in water-stressed tissues probably explains the tendency toward downward colonization in FHB.

In our experiments, we attempted to take the pathogen diversity into account by working with different species and different strains for each species. The number of isolates we could handle was limited only by the practical constraints of working on adult plants in a greenhouse. Nevertheless, our study was not designed to draw conclusions at the species scale. *F. poae* and the *Microdochium* species are considered to be less aggressive than *F. graminearum* (2, 20), at least when tested under experimental conditions. It should be noted, however, that *Microdochium* sometimes dominates in the pathogen population (25, 26). In our experiments, *F. poae* and *M. nivale* isolates appeared to be weakly aggressive, with the amount of fungal DNA being up to 40 times lower than that observed for the other *Fusarium* isolates. Among the *F. graminearum* isolates, fg159 showed a low degree of aggressiveness, with low values of both AUDPC and fungal DNA, and was not competitive against the *F. culmorum* strains. This simply illustrates the variability between isolates of the same species, as often observed in FHB studies.

The 2010 experiment suggested that the fungi progression was not symmetrical in the spike but preferentially downwards (at least for *F. graminearum*), which is consistent with other studies (17). We then focused on the competing capacity of a challenger isolate inoculated above a competing isolate. The most striking result was that the challenger isolate was able to colonize the spike only when the competitor was poorly developed. This did not depend on the species. Whether the competitor was an *F. graminearum* or an *F. culmorum* isolate did not change the outcome of the competition. Even when the competitor was a weakly aggressive isolate, its presence in the lower part of the spike still had an apparent effect on the challenger colonization (Fig. 2c). In another competition study (9), the authors also concluded that the fungal biomass in single- and mixed-species inoculations suggests that species interact competitively. That study was, however, carried out with spray inoculation and was not able to establish when the competition took place: during spore germination, infection, or spike colonization. On artificial medium, it has been shown that *F. graminearum* isolates of different vegetative groups are incompatible and form thick barrage zones at their junction, whereas subcultures of the same isolate had no visible reaction (27). This reinforces the idea that interactions of isolates of different species are competitive, leading to mutual exclusion at the scale of individual spikes.

A previous experiment using DNA mixtures at known concentrations from different *Fusarium* and *Microdochium* species indicated that PCR amplification of small amounts of target DNA from a given species may be affected by the presence of a large amount of DNA from a closely related species. We then cannot exclude a slight overestimation of the amount of DNA quantified for the species present at a low frequency on a spike. However, the possibility that the low frequency of one of the species in a coinoculated spike might even been lower does not change our conclusion of a clear exclusion of one of the competing species.

Population studies show that the coexistence of fungal species of the FHB complex is frequent in field epidemics (2). Nielsen et al. (25) found up to seven different *Fusarium* species, along with *Microdochium* spp., in grain samples, confirming the existence of a diverse *Fusarium* species complex causing the FHB epidemics in the field. Audenaert et al. (6) recorded up to nine *Fusarium* spp. in addition to *M. nivale* in a grain sample and found clear associations between *F. poae* and *F. avenaceum*, as well as between *F. graminearum* and *M. nivale/majus*. Xu et al. (4) established significant positive interactions among species of the FHB complex on the scale of four European countries. The same authors later confirmed this result (15) but attributed the positive association among species to similar responses to climatic conditions rather than to direct synergy. Although FHB species frequently cooccur in the same field, our results suggest that one species tends to dominate in a single spike. Investigations of the local distribution on individual host plants of the *Fusarium* and *Microdochium* species in field epidemics would be useful to confirm the results presented here.

ACKNOWLEDGMENTS

We are grateful to Bayer CropScience for their financial support and to the Ile-de-France region for their contribution to the UPLC-UV-MS/MS acquisition. Financial support was also provided by the ANR DON&co project.

A part of the isolates was supplied by INRA MycSa Bordeaux. We thank M. Dufresne for her help in the methodological developments and B. Beauzoone and M. Willigsecker for their technical support. The PCR tools were developed by S. Elbelt.

We are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this article.

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