Interactions between Head Blight Pathogens: Consequences for Disease Development and Toxin Production in Wheat Spikes

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Head blight (HB) is one of the most damaging diseases on wheat, inducing significant yield losses and toxin accumulation in grains. Fungal pathogens responsible for HB include the genus *Microdochium*, with two species, and the toxin producer genus *Fusarium*, with several species. Field studies and surveys show that two or more species can coexist within a same field and coinfect the same plant or the same spike. In the current study, we investigated how the concomitant presence of *F. graminearum* and another of the HB complex species influences the spike colonization and the toxin production by the fungi. To study these interactions, 17 well-characterized isolates representing five species were inoculated alone or in pairs on wheat spikes in greenhouse and field experiments. The fungal DNA in the grains was estimated by quantitative PCR and toxin contents (deoxynivalenol and nivalenol) by ultraperformance liquid chromatography-UV detection-tandem mass spectrometry. The responses of the different isolates to the presence of a competitor were variable and isolate specific more than species specific. The development of the most aggressive isolates was either unchanged or a slightly increased, while the development of the less aggressive isolates was reduced. The main outcome of the study was that no trend of increased toxin production was observed in coinoculations compared to single inoculations. On the contrary, the amount of toxin produced was often lower than expected in coinoculations. We thus conclude against the hypothesis that the co-occurrence of several HB-causing species in the same field might aggravate the risk linked to fusarium toxins in wheat production.

In addition to causing yield losses in wheat and other cereals, *Fusarium* species pose a serious threat to food safety because of their ability to produce mycotoxins. The most frequent toxin producer is *Fusarium graminearum*, which is able to produce deoxynivalenol (DON) or nivalenol (NIV). European regulation imposes a regulatory threshold on the DON toxin content above which wheat grain cannot be sold. In this context, a specific concern has been raised by the fact that several species causing head blight (HB) often co-occur in the same field (1, 2, 20), including toxin producers (*Fusarium* species) and non-toxin producers (*Microdochium* species). Should greater toxin contents be expected in wheat grain in the case of coinfection by several species? Is the presence of *Microdochium* spp. a factor aggravating the risk posed by *Fusarium graminearum*? These questions represent a major concern for advisors and producers. They also raise the more fundamental matter of species coexistence when competing for the same resource. We examine here the competition of *F. graminearum* isolates with isolates of other *Fusarium* and *Microdochium* species, and we test the hypothesis that coinfection of a wheat spike by two species increases the toxin production.

Most studies on interactions among *Fusarium* species were carried out under *in vitro* conditions (3, 4) and do not allow drawing a clear conclusion. Only a few ones were done *in planta*, but most often on seedlings. Simpson et al. (3) studied interactions between *Fusarium culmorum*, *Microdochium nivale*, and *Microdochium majus in vitro* and on wheat seedlings under different temperature regimes. The outcome of the competition depended on the temperature and the sequence in which the species were inoculated and the coinoculation led to a decrease in DON production. In another *in vitro* study, Cooney et al. (4) showed that DON production by *F. graminearum* was reduced in the presence of *F. subglutinans*. Velluti et al. (5) inoculated maize grains *in vitro* with single isolates of three *Fusarium* species and under different water

and temperature conditions and showed that toxin production was enhanced or reduced, depending on the species in presence.

To our knowledge, only one study examined the competition of *Fusarium* species on wheat spikes (6). Interactions between single isolates of *F. graminearum*, *F. poae*, *F. culmorum*, and *F. avenaceum* were compared after inoculation on wheat spikes in controlled conditions. In all cases, *F. graminearum* was slightly affected or not affected by its competitor whereas the other species tended to decrease. A striking result was that the mycotoxin productivity (amount of toxin produced per unit of fungal DNA) appeared to increase considerably in coinfections compared to single inoculations.

A few studies performed in field conditions did not confirm this result. Miedaner et al. (7) found that interactions between four isolates of *F. culmorum* led to reduced visual symptoms and toxins levels, excepted when two highly aggressive isolates were inoculated together, which led to higher DON contents. In other field experiments and with different species, Reid et al. (8) and

TABLE 1 Isolates used in the 2010 and 2011 greenhouse experiments and in the 2011 field experiment[a]

Code	Species	Chemotype[b]	Avg aggressiveness[c]	Greenhouse 2010	Greenhouse 2011	Field (2011)
fg91	*F. graminearum*	NIV	+ +	×	×	×
fg159	*F. graminearum*	DON	+	×	×	
fg165	*F. graminearum*	DON	+ +	×	×	
fg178	*F. graminearum*	DON	+ + +	×	×	×
fg201	*F. graminearum*	DON	+ + +	×		
fc124	*F. culmorum*	DON	+ + +	×	×	×
fc233	*F. culmorum*	DON	+ + +	×	×	
fc129	*F. culmorum*	NIV	+ +	×	×	
fc337	*F. culmorum*	NIV	+ + +	×	×	×
fp3	*F. poae*	NIV	+		×	×
fp4	*F. poae*	NIV	+	×		
fp6	*F. poae*	NIV	+	×	×	
fp14	*F. poae*	NIV	+	×		
mm58	*M. majus*		−	×	×	
mm221	*M. majus*		−	×		
mm229	*M. majus*		−	×		
mn227	*M. nivale*		−	×	×	

[a] In the coinoculations, each *F. graminearum* isolate was inoculated in a 50:50 mixture with each of the *F. culmorum*, *F. poae*, or *Microdochium* isolates.

[b] That is, the main toxin produced.

[c] The average aggressiveness was measured on wheat spikes in other experiments. +, ++, and +++ indicate low, moderate, and high aggressiveness, respectively, as determined by AUDPC measurements on wheat spikes in the greenhouse experiment; −, not tested. Because *Microdochium* isolates were inoculated by a different method, their aggressiveness cannot be directly compared to that of the *Fusarium* isolates.

Picot et al. (9) showed that DON productivity did not vary much or was reduced in maize ear coinoculations of *F. graminearum* with *F. verticillioides* compared to single inoculations.

A major problem, when investigating the competition of HB-causing pathogens, is the ability to monitor the amount of each species, together with the toxin production on the same host individual. In an earlier study (6), as in most other studies on *Fusarium*, the toxin and DNA contents were measured on pooled samples, either for cost or technological limitations. It thus remains difficult to clearly relate toxin content and fungal DNA within an individual host unit (here, a spike). This is, however, the only way to clarify the question of how coinfections influence toxin production in wheat HB.

In the present study, we investigated how the concomitant presence of *F. graminearum* with another species causing wheat HB influences the spike colonization and the toxin production by the fungi. To this aim, we monitored the fungal development and the toxin production in individual wheat spikes in controlled conditions after single and mixed inoculations of *F. graminearum* with *F. culmorum*, *F. poae*, *M. majus*, and *M. nivale*. The use of precise quantification techniques (quantitative PCR [qPCR], liquid chromatography, and mass spectrometry) allowed quantifying both fungal DNA and toxin contents on the same individual spikes. The results were compared to those of a field experiment.

MATERIALS AND METHODS

Overview. A set of 17 isolates belonging to five species was used in a greenhouse experiment carried out in 2010 and done again in 2011. The disease severity, the fungal colonization, and the toxin production were assessed in single and in coinoculations of these species on adult wheat plants. A subset of the isolates was used for performing a similar experiment in the field conditions. In the coinoculations, each isolate of *F. graminearum* was confronted to isolates of the other species. In addition,

each isolate was inoculated alone in order to evaluate its aggressiveness level.

Plant material. For the greenhouse experiment, seeds of winter wheat cv. Royssac, considered to be highly susceptible to HB, were sown in Jiffy peat pots 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. Then, they were individually transplanted into pots filled with 1 liter of commercial compost (Klasmann peat substrate 4; Klasmann France SARL, France) with 2 g of slow-release fertilizer (Osmocote Exact 16-11-11N-P-K 3MgO Te). Pots were then placed in a greenhouse compartment at 15 to 20°C and under 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. In each pot, two or three tillers were maintained. For each spike, the flowering date was recorded. The plants were sprayed during growth with Metrafenone (Flexity at 1 ml liter^{-1}; Bayer CropScience, Germany) and Lambda-cyhalothrine (Karaté Zéon at 0.2 ml liter^{-1}; Syngenta Agro S.A.S., France) as a preventive measure to control powdery mildew (*Blumeria graminis*) and insects, respectively. These pesticides have been tested in preliminary experiments and have no detectable effect on HB. For each treatment (single inoculations and mixed inoculations), five pots with two or three spikes per pot were inoculated. The pots were randomized in the greenhouse.

For the field experiment, the same wheat cv. Royssac was sown at 275 kernels per square meter in Grignon (Yvelines, France) on 28 October 2011. Plants were grown in microplots (nine wheat rows, 1 m in length) laid out as a randomized block design with 12 treatment combinations (five inoculations with individual isolates, six coinoculations and a noninoculated control) and four replicates. Each plot was surrounded on its four sides by border plots of another variety to minimize the impact of unwanted cross-inoculations.

Fungal material. The list of fungal isolates used in the experiments is given in Table 1. The 17 isolates belong to a larger set of isolates that were preliminary characterized for their chemotypes (on grain), for their *in*

TABLE 2 Primer and probe sequences and qPCR amplification conditions for each *Fusarium* and *Microdochium* species and wheat

Target species	Name	Sequence	Reporter/quencher[a] (5′/3′)	Final concn (nM)	Annealing temp (°C)
Fusarium graminearum (EF1α)	EF1-FCFG_F	TCGATACGCGCCTGTTACC		300	62
	EF1-FG_R	ATGAGCGCCCAGGGAATG		300	62
	grami2-EF1_rev	AGCCCCACCGGGAAAAAAATTACGACA	6-FAM/TAMRA	100	62
Fusarium culmorum (EF1α)	EF1-FC_F2	CGAATCGCCCTCACACG		300	62
	EF1-FC-R2	GTGATGGTGCGCGCCTAG		300	62
	culmo2-EF1-R2	ATGAGCCCCACCAGAAAAATTACGACAA	6-FAM/TAMRA	100	62
Fusarium poae (EF1α)	EF1-FP2_F	CTCGAGCGATTGCATTTCTTT		300	60
	EF1_FP2_R	GGCTTCCTATTGACAGGTGGTT		300	60
	EF1-FP	CGCGAATCGTCACGTGTCAATCAGTT	6-FAM/TAMRA	100	60
Microdochium majus (β tubulin)	MN_Btub_F	TCAATAACCGACTCGCACTTCTT		300	60
	MN_Btub_R	GGTGCCGTTGTAGCTATTGACA		300	60
	Mmajustub	AGGCCACTTCCCTGATGGCGTATCA	6-FAM/TAMRA	200	60
Microdochium nivale (β tubulin)	Mniv_Btub_F	TCTACTTCAACGAGGTATGTCACCAT		300	62
	Mniv_Btub_R	CCTAAGTTATGTGGGTGGTCAGTTAG		300	62
	Mniv_Btub	TTCGGGCTTCACACATTCGGCC	6-FAM/TAMRA	150	62
Triticum aestivum (wheat)	Hor1f	TCTCTGGGTTTGAGGGTGAC		250	62
	Hor2r	GGCCCTTGTACCAGTCAAGGT		250	62

[a] 6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

vitro growth rates and sporulation capacities (in petri dishes), and for their *in planta* aggressiveness (HB severity on spikes, grain weight, fungal biomass, and toxin production in grains). The isolates chosen presented different level of aggressiveness (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 were all taken from field samplings in France.

The isolates were grown in petri dishes on potato dextrose agar (PDA, 39 g liter^{-1}) at 19°C and kept in the light during 3 days. For *Fusarium*, four mycelial plugs were then transferred in 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$_4$NO$_3$, and 0.5 g of KH$_2$PO$_4$ per liter) with continual shaking. After 3 days, the medium was filtered through cheesecloth to collect the spores. For *Microdochium*, fungi were transplanted twice in petri dishes with PDA and kept at 19°C in the light during 5 days. Then, 5 ml of sterile water was added into the petri dish to collect the spores. For each isolate, a spore suspension in sterile-distilled water was adjusted to a content of 2×10^4 conidia ml^{-1} using a Malassez cell. For the coinoculations in a greenhouse, mixed inocula were prepared by mixing two spore suspensions at 4×10^4 spores ml^{-1} resulting in 2×10^4 spores ml^{-1} of each isolate. The suspensions were then stored at 4°C until they were used for inoculations, which took place within the same day. After inoculation, a few microliters of each spore suspension was deposited on PDA to check for spore viability.

Inoculation procedure. In the greenhouse, the spikes were inoculated with either single isolates or a mixture of two isolates at the midflowering stage. In the coinoculation, each *F. graminearum* isolate was opposed to each isolate from the other species (Table 1). Ten (in 2011) or fifteen (in 2010) replicates were performed for each pair of fungal isolates. The inoculations were performed with an atomizer (Ecospray; Prolabo, France). An inoculation consisted in spraying each side of the entire spike with 1 ml of inoculum suspension. Due to very low infection efficacy, *Microdochium* isolates were inoculated differently, by injection of the spore suspension in each spikelet along the spike. After the inoculation, the wheat heads were enclosed during 3 days in a transparent polyethylene bag to maintain 100% humidity and promote infection.

In the field, the five central rows of each plot were inoculated with 60 ml of spore suspension at the midflowering stage with a portable sprayer (five inoculations with individual isolates, six coinoculations and a noninoculated control; Table 1). Coinoculations were done by successively spraying a spore suspension for a *F. graminearum* isolate (fg91 or fg178) and a *F. culmorum* (fc124 or fc337) or a *F. poae* isolate (fp3). A mist irrigation was applied for 2 h after inoculation and then every morning and evening of the three following days.

Disease assessment, fungal DNA, and mycotoxin quantification. In the greenhouse, the number of spikelets with HB symptoms was visually assessed thrice a week from 5 days after inoculation (appearance of first symptoms) to 28 days after inoculation. Since the aspect of HB symptoms may vary, each symptomatic spikelet showing a premature bleaching or brown necrosis was putatively considered diseased. The percentage of diseased spikelets in each spike was calculated. The dynamics of the appearance of symptomatic spikelets on each spike was characterized by the area under the disease progression curve (AUDPC), which was normalized by dividing the AUDPC value by the number of observation days. At maturity, the spikes were cut and hand-threshed to separate the kernels from the chaff. Kernels were then weighed to estimate the thousand kernel weight (TKW). Then, the kernels of each spike were ground with a mixer mill (MM 400; Retsch, France) and stored for fungal DNA and toxin quantification.

In the field, HB was visually assessed once when symptoms were the most visible (33 days after inoculation). Disease incidence was estimated as the percentage of blighted spikes in each microplot. At maturity, all the spikes along three central rows per plot were harvested and hand-threshed. From these samples, the TKW was assessed, and 200-g subsamples were ground with a mixer mill and stored for fungal DNA and toxin quantification.

The methods for species-specific quantification of fungal DNA (extraction and qPCR) and toxin quantification (extraction and ultraperformance liquid chromatography-UV detection-tandem mass spectrometry [UPLC-UV-MS/MS] analysis) from all the tissue samples were developed in a previous study (10). The total DNA was extracted from around 50 mg of ground material using the DNeasy plant minikit (Qiagen). The amount of both fungal and wheat DNA was estimated by qPCR using species-
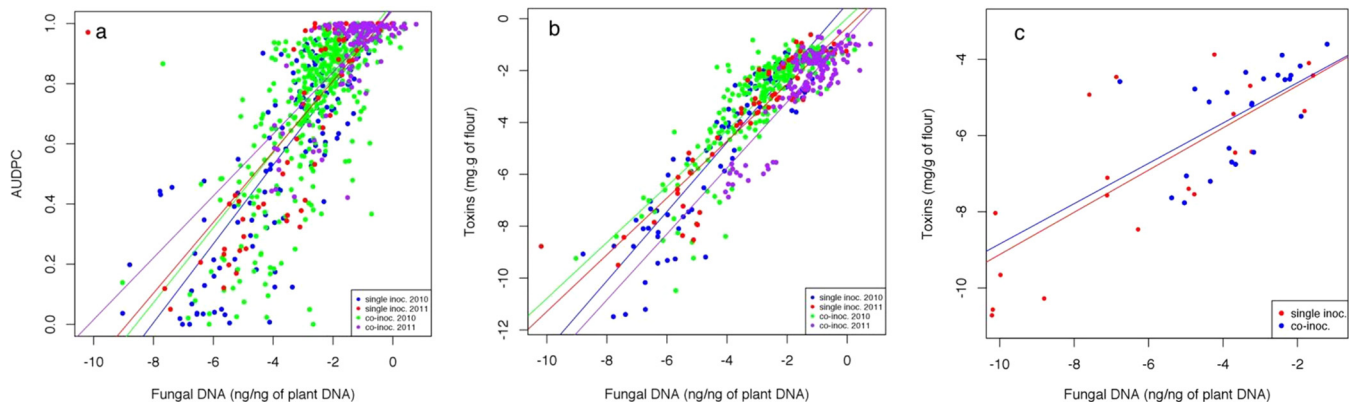
FIG 1 Relationship between AUDPC and fungal DNA and between toxin concentrations and fungal DNA in wheat samples inoculated by different species responsible for head blight in a greenhouse (a and b) or field (c) experiment. Regression lines were drawn separately for single inoculations and coinoculations in each experiment. The fungal DNA was quantified by qPCR. The toxin concentration was determined by using UPLC-UV-MS/MS. Fungal DNA and toxin concentration data are log transformed.

specific primer pairs and species-specific TaqMan probes (Table 2; see also "Extraction and quantification of fungal DNA" in the supplemental material). The amount of fungal DNA was calculated from Cq values using the standard curves from *F. graminearum*, *F. culmorum*, *F. poae*, *M. majus*, and *M. nivale*, and these values were normalized with the estimated amount of plant DNA of the same sample, quantified by wheat-specific qPCR assay. The quantification of the plant DNA was performed using plant EF1 qPCR primers and SYBR green I (10, 11) (Table 2). A homogenized flour sample of around 1 g was used for toxin extraction. The UPLC-UV-MS/MS analysis of the toxin extract was performed with an Acquity Ultra-Performance liquid chromatograph coupled with a TQD tandem mass spectrometer (Waters Corp.), as described by Siou et al. (10). The mass spectrometer was operated in electrospray ionization (ESI negative-ion mode) under multiple-reaction monitoring mode. As confirmed by single inoculations, the isolates produced either DON or NIV, according to their chemotype. As established in other studies (see, for example, references 6 and 10), the amount of acetylated derivatives were expected to be highly correlated with the amount of the predominant DON or NIV toxin and only those two were quantified. For cost limitation, only eight replicates (spikes) were used for toxin quantification.

Data analyses. An objective of this study was to assess the impact of species interaction on the amount of toxin produced. To this end, the toxin content in the grains was compared between spikes inoculated with a single species and spikes coinoculated with a mixture of two species. An expected toxin content was calculated for the coinoculations, based on the respective species-specific fungal DNA that were estimated by qPCR and on the toxin production in spikes inoculated with a single species. For each isolate, the expected toxin content was calculated as (TOX.S/DNA.S) × DNA.C, with TOX.S and DNA.S as the average toxin and fungal DNA contents, respectively, in single inoculations and DNA.C as the species-specific fungal DNA produced by each isolate in coinoculations. The expected toxin contents accounted for by each isolate was then added to give the total expected content in the spike. This expected toxin content was compared to the measured toxin content to establish whether the presence of another species in the same spike enhanced or reduced the toxin production. For each pair of isolates, we evaluated whether the difference between expected and measured toxin contents was significantly different from zero with Wilcoxon's tests. In order to produce synthetic results, the percentage of spikes for which the difference between measured and expected toxin contents was negative, null, or positive was established by pooling the data for each competing species and for the 2010 and 2011 experiments.

Analyses of variance (ANOVAs) were performed with the DON and NIV concentrations, the quantities of fungal DNA, and the HB severity (AUDPC) as variables to be explained and the year of experiment and the

isolate as explanatory variables for the 2010 and 2011 greenhouse experiments. Multiple comparisons were done with the Tukey's honestly significant difference (HSD) test. Residuals distribution was checked for variance homogeneity and normality. Regression analyses were used to investigate the relationship between variables such as AUDPC and fungal DNA or fungal DNA and toxin contents.

RESULTS

Correlations between variables. In the greenhouse experiment, the AUDPC was found highly correlated to the fungal DNA, as

TABLE 3 ANOVA results with the toxin concentration, the quantities of fungal DNA, and the HB severity as variables to be explained and the year of the experiment and the isolate as explanatory variables for the 2010 and 2011 greenhouse experiments[a]

Source of variation[b]	df	MS	F-value	P
On toxin concentrations				
Exp (A)	1	0.552	135.56	<0.001
Iso (B)	69	0.083	20.34	<0.001
A × B	32	0.028	6.86	<0.001
Residuals	615	0.004		
On fungal DNA				
Exp (A)	1	5.362	143.15	<0.001
Iso (B)	73	0.127	3.38	<0.001
A × B	34	0.144	3.84	<0.001
Residuals	1,336	0.037		
On AUDPC				
Exp (A)	1	1.730	90.96	<0.001
Iso (B)	73	0.846	44.51	<0.001
A × B	34	0.239	12.55	<0.001
Residuals	1,334	0.019		

[a] ANOVA was performed with the toxin concentration (either DON or NIV, depending on the isolates), the quantities of fungal DNA and the HB severity (AUDPC) as variables to be explained and the year of experiment (Exp) and the isolate (Iso) as explanatory variables for the 2010 and 2011 greenhouse experiments. In the coinoculation, each *Fusarium graminearum* isolate (fg91, fg159, fg165, and fg178) was opposed to each isolate of *F. culmorum* (fc124, fc129, fc233, and fc337), *F. poae* (fp3, fp4, fp6, and fp14), *Microdochium majus* (mm58, mm221, and mm229), and *Majus nivale* (mn227) (see Table 1). MS, mean square.

[b] Exp (A), year of experiment (2010 or 2011); Iso (B), either a single isolate or pair of isolates.
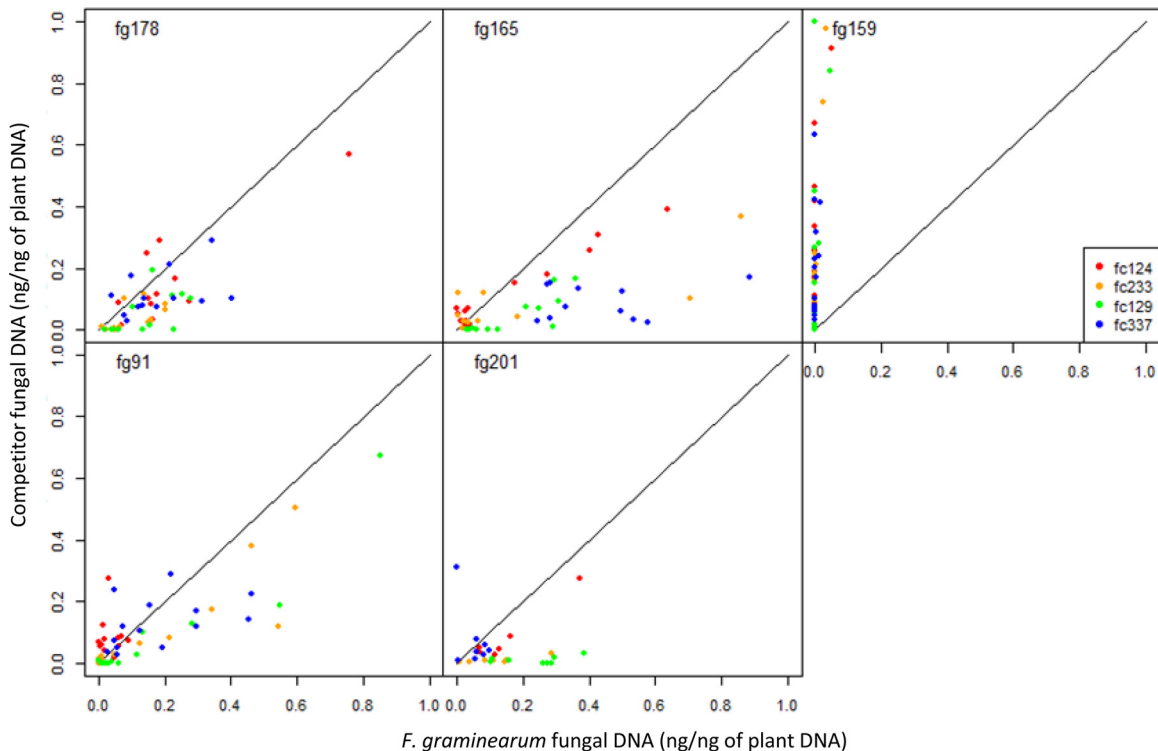
FIG 2 Amounts of DNA of *F. culmorum* (on the ordinate) plotted against those of *F. graminearum* (on the abscissa) in each individual wheat spike in the 2010 and 2011 greenhouse experiments. Each graph corresponds to an *F. graminearum* isolate, and each color indicates its *F. culmorum* competitor. fg178 produced more DNA than the competing isolate in 69/75 of the spikes in 2010 and 26/30 in 2011. fg165 produced more DNA than the competing isolate in 50/65 in 2010 and in every spike in 2011. fg201 produced more DNA in 71/78 spikes in 2010, and fg91 produced more DNA in 50/85 spikes in 2010 and in 33/35 in 2011. Additional tables (means values for fungal DNA quantified in wheat spikes inoculated with different *Fusarium* and *Microdochium* isolates in the 2010 and 2011 experiments) are presented in Table S1 in the supplemental material.

expected ($r = 0.78$, $P < 0.001$; Fig. 1a, all values pooled). As well, the toxin content was found highly correlated to the fungal DNA ($r = 0.82$, $P < 0.001$; Fig. 1b, all values pooled). In the field experiment, a similar correlation was found between the fungal DNA and the toxin contents ($r = 0.78$, $P < 0.001$; Fig. 1c, all values pooled).

Effect of coinoculations on disease severity. In both the 2010 and the 2011 greenhouse experiments and whatever the isolate considered, AUDPC was highly variable. An ANOVA analysis showed a significant effect of the year experiment ($P < 0.001$) that explained 61% of the variance in AUDPC (Table 3). The AUDPC was lower in 2010 (0.65 in average) than in 2011 (0.74 in average), except for fc337, for which it was the opposite in coinoculations and single inoculations. The isolate (or pair of isolates) significantly influenced the AUDPC. Multiple mean comparisons were performed separately for each year and showed that *F. poae* isolates and fg159 were always less aggressive than the other isolates (Tukey's HSD test, $P < 0.001$). Since *Microdochium* isolates were inoculated with a different method, their AUDPC values cannot be directly compared to those of the *Fusarium* isolates.

In the field experiment (Fig. 2), fp3 and fc337 developed less symptoms than the three other isolates (fg178, fg91, and fc124) when inoculated alone. Coinoculation with other isolates did not significantly affect severity compared to single inoculations with *F. graminearum* isolates.

Effect of coinoculations on fungal DNA. In the coinoculations, the fungal DNA of each species was expected to decrease

relative to single inoculations since both isolates had to share the same spike, viewed as a limited resource, but this was not always the case. For instance, the fungal DNA of fc124 was significantly greater when it was inoculated with fg159, compared to single inoculations, in the 2010 experiment (Table S1).

The experiment (year) significantly influenced the amounts of fungal DNA measured ($P < 0.001$; Table 3). For instance, in 2011, both *Microdochium* isolates mm58 and mn227 produced more fungal DNA when inoculated with fg178 than when inoculated alone, while this was not the case in 2010: mm58 produced less fungal DNA in the presence of fg178, and there was no significant difference for mn227 (see Table S1 in the supplemental material). More generally, depending on the isolates and on the year, the fungal DNA increased, decreased or remained stable in the coinoculations compared to the single inoculations (see Table S1 in the supplemental material). Several isolates appeared better competitor than others. In most cases, the fungal DNA of isolates fg159, fp6, and fp14 was less when confronted to a competitor. On the contrary, the DNA of fg201 was higher when confronted to a competitor. The situation was less clear with fg165, which seemed to be a good competitor in 2010 but not in 2011; as well, fg178 seemed to be a good competitor in 2011 but not in 2010. When fg159 was inoculated with another poorly aggressive isolate (such as fp3, fp4, fp6, or fp14), its development was not significantly affected. In general, it was difficult to infer any general trend from these data, regarding the outcome of isolate competition for spike colonization.
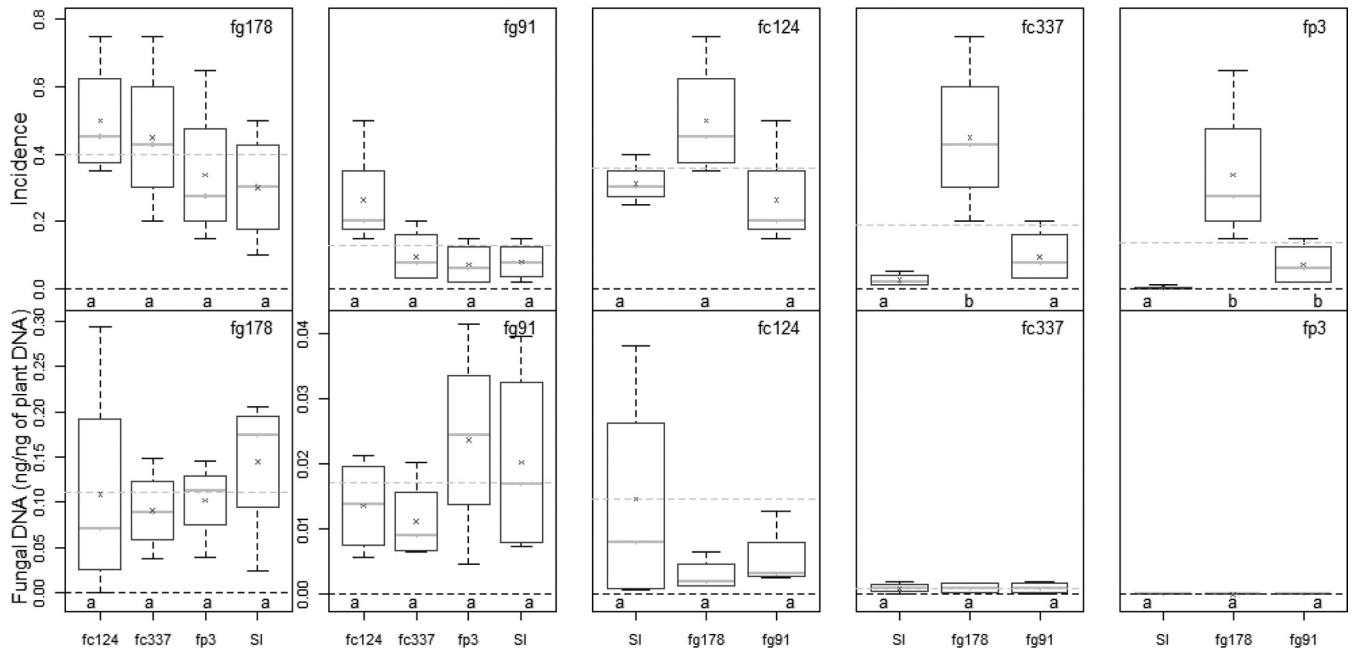
FIG 3 Variations of HB severity (percentage of blighted spikes) and fungal DNA in field microplots inoculated with five isolates belonging to three *Fusarium* species (*F. graminearum*, *F. culmorum*, and *F. poae*; see Table 1) in single inoculations (SI) or in coinoculation. Treatments indicated by the same lowercase letter within the same graph were not significantly different; the six coinoculation treatments (fg178/fc337, fg178/fc337, fg178/fp3, fg91/fc337, fg91/fc337, and fg91/fp3) are presented twice in order to compare each of them with the two single inoculations (fg178 and fg91).

The difficulty to draw conclusion from the above analysis partly came from the high variability in the fungal colonization among the different replicates. We thus performed a different analysis, based on the direct comparison of the fungal DNA of each isolate within each spike. Figure 2 shows the amounts of DNA of *F. culmorum* plotted against those of *F. graminearum* in each individual spike. This gives a much clearer picture of the overall effect of coinoculation on spike colonization by each species. Only *F. graminearum* and *F. culmorum* isolates were considered in this analysis because the others isolates had a low aggressiveness (Table 1). A Wilcoxon test indicates that the *F. graminearum* isolates, except fg159, produced in average more fungal DNA than their competing *F. culmorum* isolates ($P <$ 0.001). This analysis, based on the comparison of DNA amounts at the scale of single spikes, revealed that some isolates, such as fg178 and fg165, were better competitor than others, whereas fc129 and fc337 tended to be poor competitors (Fig. 2). In the field experiment, no significant difference in fungal DNA was observed between coinoculations and single inoculations (Fig. 3).

Effect of coinoculations on toxin production. The amount of toxins produced (whether it was DON or NIV) depended on the year of experiment, the isolates and their interaction (Table 3). Since the toxin content in the spikes was highly correlated with the fungal DNA (Fig. 1), it is likely that the dependency of toxin content to the environmental effects was largely accounted for by differences in the fungus development.

The greenhouse experiments did not reveal an increase in the toxin productivity in coinoculations, relative to single inoculations. The situations in which the toxin production was similar or lower in coinoculations were the most frequent (Fig. 4). In the greenhouse experiments, whatever the year and the isolate combination, significantly less DON was produced than expected in

coinoculations for 57.4% of the spikes. The same trend was observed for NIV, with 64.2% of the spikes having a lower toxin content than expected. Figure S1 in the supplemental material shows the data in more detail: in the 2010 greenhouse experiment, the toxin productivity increased moderately in some spikes but remained unchanged or decreased in the majority of the replicates, and in 2011 the toxin productivity was either lower or unchanged in coinoculations relative to single inoculations. In the field experiment, expected and measured toxin values were not found significantly different.

DISCUSSION

The main outcome of our study is that toxin productivity did not increase in coinoculations, relative to single inoculations. On the contrary, it was significantly reduced for several isolate combinations. This was clearly shown by analyzing each spike individually for both toxin production and fungal DNA. This result is confirmed by the fact that the relationship between toxin content and fungal DNA is linear and mainly accounted for by the quantity of DNA. The same conclusion was reached from the greenhouse experiment and from the field experiment.

This result is in accordance with other studies (7–9) that were realized in field conditions. Although these studies did not allow researchers to precisely relate fungal colonization and toxin production within the same host plant, the findings suggest that strain competition does not involve a significant increase in toxin production in natural conditions. Our own field experiment reaches the same conclusion and is in good accordance with the results obtained in the greenhouse experiments. Note, however, that competition was evaluated here in wheat heads but certainly takes place in other environments (e.g., crop debris), where the interactions and role of toxins may be different. *In vitro* studies (3, 12)
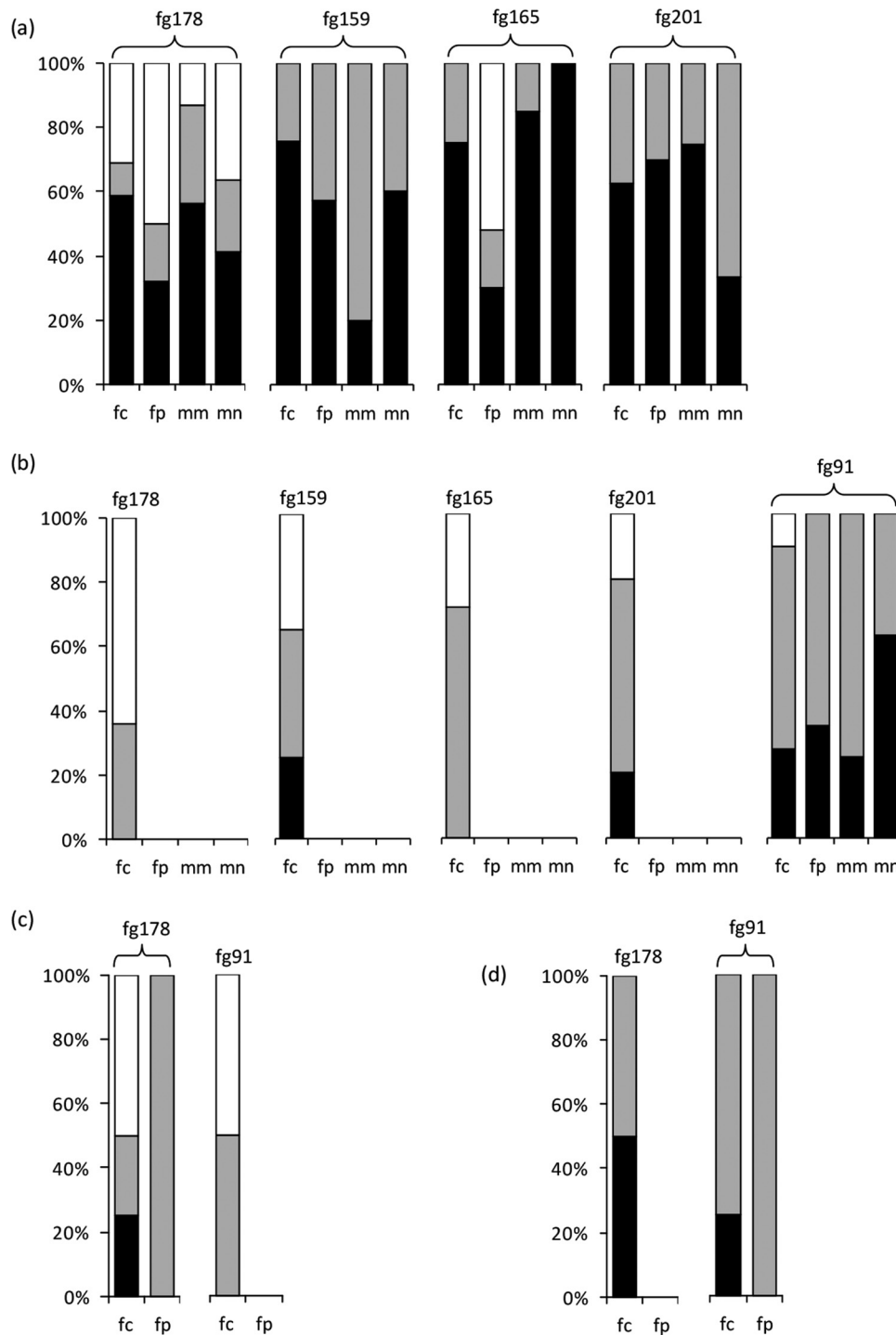
FIG 4 Differences between measured and expected contents of DON (a, greenhouse experiments; c, field experiment) and NIV (b, greenhouse experiments; d, field experiment) in individual wheat spikes in which *Fusarium graminearum* was coinoculated with *F. culmorum*, *F. poae*, or *Microdochium nivale*. Black, gray, and white bars indicate the percentages of spikes in which the measured toxin content was significantly lower, not different, or higher, respectively, than the level expected from single inoculation data (Wilcoxon test). The figures synthesize the coinoculation data for the 2010 and 2011 greenhouse experiments (DON; isolates fc124, fc129, fc233, fc337, fp3, fp4, fp6, fp14, mn58, mn221, mn227, and mn229) (a), the 2010 and 2011 greenhouse experiments (NIV; isolates fc124, fc129, fc233, fc337, fp4, fp6, fp14, mn58, mn221, mn227, and mn229) (b), the 2011 field experiment (DON; isolates fc124, fc337, and fp3) (c), and the 2011 field experiment (NIV; isolates fc124, fc337, and fp3) (d). The *F. graminearum* isolates are indicated on the graphs. Values for individual spikes are also presented in Fig. S1 in the supplemental material.

also suggest that toxin production is proportional to the quantity of fungal DNA and does not increase when strains are in competition. The study from Xu et al. (6) yielded, however, a different conclusion and showed a strong increase in toxin productivity in the case of coinoculations. By working with several isolates belonging to four HB species and by measuring toxin contents in each spike, we never found such a strong increase in DON or NIV productivity in the case of coinoculations. In 2011, we only observed a slight but significant increase in the coinoculations (see, for instance, fg178). The increase in toxin productivity established by Xu et al. (6) may have resulted from specific conditions, such as high temperatures during initial infection periods. It may also be due to an isolate-specific response (the authors of that study only examined one isolate per species).

Our study was not designed to draw conclusions at the species scale. We simply 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 only limited by the practical constraints of working on adult plants in a greenhouse. We can nevertheless compare our results with published data on HB species. *F. poae* is generally believed to be less aggressive than the other *Fusarium* species (13, 14), which was found again in our experiment. We found that *F. poae* produced a lower disease severity and smaller amounts of fungal DNA in the grains compared to other *Fusarium* species in both greenhouse and field experiments. In contrast, no significant difference was found between *F. graminearum* and *F. culmorum* isolates for spike colonization, except for fg159, which showed a low aggressiveness and was not competitive against the *F. culmorum* strains. This illustrates the variability between isolates of the same species, as often observed in FHB studies.

Both our field and greenhouse experiments showed that isolates with a low aggressiveness are poor competitors against aggressive isolates, whereas the development of aggressive isolates (such as fg178, fg201, fc124, and fc233) was not or slightly altered by the presence of a poorly aggressive isolate. This is consistent with (6) and with the results of Velluti et al. (5), which established that the growth rate of *F. graminearum* was relatively unaffected by the presence of less aggressive species (*F. proliferatum* and *F. moniliforme*).

Environmental factors are known to influence the development of plant pathogens (15), and environmental effects are commonly found in experiments dealing with *Fusarium* and *Microdochium* (16, 17). This might explain the differences between the 2010 and the 2011 experiments. In 2010, the DON and NIV productivities in coinoculations were either lower or not significantly different from that in single inoculations, whatever the isolate combination. In 2011, even if most isolate combinations responded as in 2010, the toxin productivity sometimes slightly but significantly increased in the coinoculations (see, for instance, fg178).

For practical reasons, only one host variety could be used in our study. We cannot exclude an influence of the host genotype on species competition and the resulting toxin productivity, but this should be assessed in further experiments. Note, however, that in a different species (maize), Reid et al. (8) and Picot et al. (9) also found that DON production did not vary much or was reduced in coinoculations compared to single inoculations.

In the greenhouse experiments, the correlation between the fungal biomass (estimated by DNA quantification) and HB sever-

ity (AUDPC) was strong and positive, in agreement with other studies using similar techniques (6, 18, 19). A good correlation between fungal biomass and HB severity is expected when the inoculations are performed at the most receptive stage of the host plant, which was the case in both our greenhouse and our field study.

In most cases, the HB severity increased or remained stable in the coinoculations compared to the single inoculations. This is consistent with other studies (see, for example, reference 6). Some exceptions were however observed in 2010, with lower AUDPC values produced by coinoculations. A similar effect was observed in the field by Miedaner et al. (7), who measured a lower HB severity and a lower yield reduction in coinoculations than in single inoculations. The outcome of HB species competitions in terms of disease severity seems difficult to predict and may depend on the strains, as well as on environmental conditions (6). In all cases, however, the disease severity was strongly linked to the fungal biomass.

Our data suggest that DON and NIV productivity either does not increase in multiple infections by two HB species on the same wheat spike or increases to a very limited extent and for some strains only. This conclusion disagrees with that of another study (6) but is in accordance with several field experiments. According to these data, the DON and NIV toxins do not seem to be a key element in the competition between HB species for host colonization.

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