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Effect of wheat infection timing on Fusarium head blight causal agents and secondary metabolites in grain

Giovanni Beccari[a], Consuelo Arellano[b], Lorenzo Covarelli[a,c], Francesco Tini[a], Michael Sulyok[d], Christina Cowger[e,*]

[a] Department of Agricultural, Food and Environmental Sciences, University of Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
[b] Department of Statistics, North Carolina State University, 27695 Raleigh, USA
[c] Centre for Crop and Disease Management, Curtin University, 6102, Kent Street, Bentley, Perth, Western Australia, Australia
[d] Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenzstrasse 20, A-3430 Tulln, Austria
[e] United States Department of Agriculture-Agricultural Research Service, Department of Entomology and Plant Pathology, North Carolina State University, 27695 Raleigh, USA

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ABSTRACT

Fusarium head blight (FHB) results in yield loss and damaging contamination of cereal grains and can be caused by several *Fusarium* species. The objective of the present study was to determine, in a greenhouse experiment on winter wheat, how FHB was affected by timing of infection (0, 3, 6 or 9 days after anthesis, daa) by the aggressive species *Fusarium graminearum* compared to the relatively weak species *Fusarium avenaceum*, *Fusarium poae* and *Fusarium acuminatum*. Measures of FHB development were: symptoms in spikes (visually assessed), fungal biomass (quantified by real time quantitative PCR) and accumulation of fungal secondary metabolites (quantified by liquid chromatography-tandem mass spectrometry) in kernels. With regard to symptoms, *F. graminearum* was unaffected by inoculation timing, while the weaker pathogens caused greater disease severity at later timings. In contrast, the accumulation of *F. graminearum* biomass was strongly affected by inoculation timing (3 daa ≥ 6 daa ≥ 0 daa = 9 daa), while colonization by the weaker pathogens was less influenced. Similarly, *F. graminearum* secondary metabolite accumulation was affected by inoculation timing (3 daa ≥ 6 daa ≥ 0 daa = 9 daa), while that of the weaker species was less affected. However, secondary metabolites produced by these weaker species tended to be higher from intermediate-late inoculations (6 daa). Overall, infection timing appeared to play a role particularly in *F. graminearum* colonization and secondary metabolite accumulation. However, secondary metabolites of weaker *Fusarium* species may be relatively more abundant when environmental conditions promote spore dispersal later in anthesis, while secondary metabolites produced by *F. graminearum* are relatively favored by earlier conducive conditions.

1. Introduction

Wheat (*Triticum* spp.) is one of the most important small-grain cereals in the world, with a production of about 730 million tonnes per year (FAO, 2017). This production may be significantly reduced by several fungal diseases worldwide. Among them, fusarium head blight (FHB) is one of the most widespread and damaging diseases, capable of strongly compromising not only crop yield but also quality. FHB is caused by many distinct species belonging to multiple *Fusarium* species complexes (O'Donnell et al., 2013). These causal agents are able to biosynthesize mycotoxins, substances with toxic activity in humans and animals (da Rocha et al., 2014).

Among the various species causing FHB, *Fusarium graminearum* is considered the most important globally due to its widespread incidence and aggressiveness (Goswami and Kistler, 2004; Kazan et al., 2012). However, other species considered "weak" pathogens (Brennan et al.,

Abbreviations: FHB, Fusarium head blight; DON, Deoxynivalenol; 15AcDON, 15 acetyl-deoxynivalenol; 3AcDON, 3 acetyl-deoxynivalenol; NIV, Nivalenol; BEA, Beauvericin; ENs, Enniatin analogues; ENB1, Enniatin B1; ENB2, Enniatin B2; ENB3, Enniatin B3; ENA, Enniatin A; ENA1, Enniatin A1; ENA2, Enniatin A2; MON, Moniliformin; GS, Zadoks Growth Stage; daa, Days after anthesis; PDA, Potato dextrose agar; dai, Days after inoculation; DS, Disease severity; q-PCR, Real time quantitative PCR; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; Ct, Cycle threshold; LOD, Limit of detection; ESI, Electrospray ionization; MRM, Multiple reaction monitoring

* Corresponding author.
E-mail address: christina.cowger@ars.usda.gov (C. Cowger).

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2003; Osborne and Stein, 2007), such as *F. avenaceum*, *F. poae* and *F. acuminatum*, may often be associated with FHB in many wheat cultivation areas across the world, and have increased in importance in the composition of the FHB complex in recent years (Beccari et al., 2018; Birzele et al., 2002; Covarelli et al., 2015a; Gräfenhan et al., 2013; Infantino et al., 2012; Ioos et al., 2004; Isebaert et al., 2009; Karlsson et al., 2016, 2017; Lindblad et al., 2013; Marín et al., 2012; Nielsen et al., 2011; Osborne and Stein, 2007; Stenglein, 2009; Tittlemeier et al., 2013; Uhlig et al., 2007; Xu et al., 2005, 2008). In addition, different regions can have different dominant FHB-causing species. For example, in a Canadian harvest sample survey from 1995 to 2008, *F. avenaceum* was the main causal agent of FHB in durum wheat in several Saskatchewan crop districts, while *F. graminearum* was the main FHB agent on durum wheat in at least two Alberta districts (Tittlemeier et al., 2013).

The various FHB causal agents also possess diverse mycotoxigenic profiles that can lead to different types of grain contamination depending on the presence and incidence of each species in the crop. For example, *F. graminearum* is the main producer of the well-studied type B trichothecenes, such as deoxynivalenol (DON), with its acetylated forms (15 acetyl-deoxynivalenol, or 15AcDON, and 3 acetyl-deoxynivalenol, or 3AcDON), and nivalenol (NIV) (Bottalico and Perrone, 2002; Geraldo et al., 2006). In addition to NIV, *F. poae* produces type A trichothecenes such as T-2 toxin and HT-2 toxin (Jestoi et al., 2008; Thrane et al., 2004), although with low frequency and quantity (Stenglein, 2009). This last species is also able to biosynthesize secondary metabolites belonging to the depsipeptide family, such as beauvericin (BEA) and enniatin analogues (ENs) (such as enniatin B1 or ENB1, enniatin B2 or ENB2, enniatin B3 or ENB3, enniatin A or ENA, enniatin A1 or ENA1, and enniatin A2 or ENA2) (Covarelli et al., 2015b; Jestoi et al., 2008). ENs and BEA are also biosynthesized by *F. avenaceum* (Jestoi et al., 2008; Vogelgsang et al., 2008), which in addition is a producer of moniliformin (MON) (Morrison et al., 2002; Yli-Mattila et al., 2006). *F. acuminatum* possesses a similar mycotoxigenic profile to *F. avenaceum*, being able to biosynthesize secondary metabolites such as ENs (Visconti et al., 1992) and MON (Schütt et al., 1998). In addition to these better-known compounds, other secondary metabolites produced by *Fusarium* species may be detected and investigated using multi-analyte methods developed in recent years based on liquid chromatography coupled to mass spectrometry (Pereira et al., 2014).

Due to their toxic activity against humans and animals, the well-studied mycotoxins in wheat grain such as DON (Commission Regulation, 2006) and T-2 and HT-2 toxins (Commission Recommendation, 2013) have had maximum or recommended levels established in the European Union. Similarly, the USA has set advisory maximum levels for DON in wheat grain and finished wheat-based products (U.S. Department of Health and Human Services Food and Drug Administration, 2010). However, other mycotoxins have not yet been regulated and the impact of many other fungal secondary metabolites on consumer health remains unclear.

In general, the spectrum of *Fusarium* species involved in FHB, their incidence within the complex, and as a result, the type and quantity of secondary metabolites accumulating in grain depend on weather conditions, especially during anthesis (Kelly et al., 2015; Oerke et al., 2010). Anthesis is considered the most susceptible growth stage for *Fusarium* infection of wheat (Parry and Nicholson, 1996; Yoshida, 2012). In particular, extruded wheat anthers are considered the primary infection site, allowing the fungal hyphae to establish infection and entry to individual florets (Brown et al., 2010; Siou et al., 2014). However, *Fusarium* can also infect wheat at other sites, such as the adaxial surface of the glumes, the lemma or the palea (Brown et al., 2010; Goswami and Kistler, 2004; Siou et al., 2014).

Timing of infection is known to be important in determining disease occurrence (Cowger and Arellano, 2010). For example, anther infection often results in lack of kernel development, while slightly later infection leads to shriveled mycotoxin-containing kernels, and even later infections can result in healthy-appearing kernels that contain mycotoxins, especially when moisture is abundant (Cowger et al., 2009; Del Ponte et al., 2007; Hart et al., 1984; Siou et al., 2014).

Previous research indicated that the period of maximum receptivity of wheat to *F. graminearum* infection was until around 10 days after mid anthesis (Cowger and Arellano, 2010). However, duration of the susceptibility period may depend on environmental conditions (Lacey et al., 1999) and cultivar (Schroeder and Christensen, 1963). For example, the occurrence of high humidity post-anthesis produced late infections, leading to kernels with a low level of symptoms but a high level of DON (Cowger and Arellano, 2010, 2013). Also, phenotypes with partially extruded anthers showed relatively more severe FHB symptoms compared with a closed-flowering phenotype, as well as with those exhibiting rapid and full anther extrusion and ejection (Kubo et al., 2013). This may be due to the fact that anthers trapped between glumes provide dead tissue readily colonized by *Fusarium* (Skinnes et al., 2010).

Taking into account these findings, the objective of the present study was to further explore the period of maximum receptivity (up to around 10 days after early anthesis) of wheat, and expand the investigation beyond *F. graminearum* to include other species that in many countries comprise the FHB complex, in particular *F. avenaceum*, *F. poae* and *F. acuminatum*. These different FHB causal agents were compared with regard to the timing of infection in wheat. Further, this study extended the investigation beyond the best-known mycotoxins to a wide range of fungal secondary metabolites accumulated in grains. Specifically, the study sought to determine how disease occurrence, fungal biomass and fungal secondary metabolite accumulation were affected in wheat by infection timing of the different FHB causal agents.

2. Materials and methods

2.1. Plant material and experimental design

Vernalized seedlings of cv. Dyna-Gro Shirley, a commercial cultivar of soft red winter wheat (*T. aestivum*) susceptible to FHB, were transplanted (Zadoks Growth Stage 05 = GS05) in 15 × 15 × 20 cm plastic pots (one seed per pot) previously filled with a 1:1 (w/w) mixture of Fafard 3 M Mix (Sungro Horticulture, Agawam, MS, USA) and sand amended with fertilizer (Osmocote, Marysville, OH, USA) (500 mg/kg). Pots were randomized and grown in a greenhouse at North Carolina State University in Raleigh, NC, USA, at a temperature of 22 ± 2 °C. Plants were regularly watered and fertilized at tillering (GS24) (using Jack's Classic Water Soluble Plant Food, J.R. Peters, Inc., Allentown, PA, USA) (300 mL/kg of 0.5 g/L solution) and heading (GS59) (Osmocote, 250 mg/kg). The entire experiment was conducted from November 2016 to March 2017.

Due to greenhouse space limitations, the experiment was conducted with two experimental replicates in time (trials). In each trial, the same four fungal strains (described below) and four inoculation timings (0, 3, 6 and 9 days after anthesis, or daa) were used. In each trial, replicate plants were subjected to each combination of fungal strain and inoculation timing (seven replicate plants in trial 1 and 10 in trial 2). Thus, a total of 112 plants were used in trial 1 and 160 plants in trial 2, for 276 plants total.

2.2. Fungal material, inoculum production and inoculation procedure

The fungal strains used in this experiment were four different causal agents of FHB: *F. graminearum* strain 38–49 (15AcDON chemotype), *F. poae* strain 30–21, *F. avenaceum* strain 29–31 and *F. acuminatum* strain 9–14. These four strains were previously isolated from soft red winter wheat spikes collected in North Carolina (USA), subjected to molecular identification and characterization (T. Ward, United States Department of Agriculture-Agricultural Research Service, personal communication, 2016) and stored at −20 °C in glycerol.

All strains used in this experiment were previously cultured at 24 °C on potato dextrose agar (PDA) (BD, Franklin Lakes, NJ, USA) in 9-cm Petri dishes. Conidial inoculum of the *F. graminearum*, *F. avenaceum*, and *F. acuminatum* strains was produced in mung bean broth. Mung beans were steeped in boiled deionized water at a rate of 40 g/L for 15 min, after which the broth was filtered through cheesecloth and autoclaved. Mycelial plugs from one-week-old *Fusarium* colonies were transferred into 2-L flasks containing 1 L of mung bean broth and then aerated with forced sterile air for 1 week at 22 °C and 12 h light. Conidial inoculum of the *F. poae* strain was obtained using V8 liquid medium, prepared by diluting V8 juice (Campbell's, Camden, NJ, USA) in deionized water (1:4 v/v) and autoclaving. Mycelial plugs from one-week-old colonies were transferred into 1-L flasks containing 500 mL of V8 liquid medium and then agitated in an orbital shaker at 22 °C and 12 h light for two weeks.

All resulting conidial suspensions were filtered through cheesecloth and the conidia collected by centrifugation at 1157 × *g* for 5 min in an Allegra X-30R centrifuge (Beckman Coulter, Brea, CA, USA). Conidial concentrations were determined using a hemocytometer and adjusted to 7×10^6 conidia/mL in deionized sterile water to obtain stock concentrations subsequently stored at −20 °C.

After thawing for use, the germinability of the cultures used in inoculation was tested and compared by diluting each suspension to 10^2 conidia/mL, inoculating three one-quarter-strength PDA plates per strain with 300 μL per plate, and incubating the plates at 25 °C. Colonies were counted after two days, and germinability of all strains was found to be within the range of 9–14% on media.

Plants were inoculated at four different timings: 0, 3, 6 and 9 daa. Zero daa (day 0) was considered to be the day on which 30–50% of the anthers on a spike were extruded (GS61). The other three inoculation timings were determined in relation to day 0 and corresponding to GS65, GS69 and GS71, respectively. For inoculation, a suspension of 1×10^6 conidia/mL amended with 0.05% of Tween 20 (Sigma Aldrich, Saint Louis, MO, USA) was used. The main spike of each pot was isolated in a plastic funnel and sprayed with a single-strain conidial suspension using an atomizer (Misto® sprayer) at the rate of 6 sprays per spike (about 4 mL), ensuring application to the entire spike surface.

Thirty minutes after inoculation, plants were placed in a mist chamber on the greenhouse bench, enclosed with plastic curtains to create high humidity. Misting was provided using nebulizers with small-diameter orifices installed about 30 cm over the spikes. By means of a programmable timer, mist was applied for 30 s in each 20 min period for 9 h during the day, followed by a night without misting. After 3 days, plants were removed from the mist chamber. Pots were watered as needed until three weeks before harvest.

2.3. FHB symptom evaluation and sample collection

On each inoculated spike, FHB symptoms were evaluated at 7, 14 and 21 days after inoculation (dai) and disease severity (DS) was determined as (number of diseased spikelets/total number of spikelets). Inoculated spikes were hand-harvested at maturity (GS92), 60 days after the 0 daa inoculation timing. Spikes were threshed in a single-spike thresher (Precision Machine, Lincoln, NE, USA), such that all the kernels were retained.

Within each trial, the kernels of the 7 or 10 spikes belonging to the same fungal strain ∗ inoculation timing treatment were bulked, finely ground by laboratory mill and divided in two subsamples. One subsample was used for real time quantitative PCR (q-PCR) analysis to determine fungal biomass (see Section 2.4), and the other for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify and quantify mycotoxins (see Section 2.5). Resources did not permit these two analyses to be performed on individual replicate spikes without bulking.

2.4. Detection and quantification of fungal biomass by q-PCR

To determine the standard curves for q-PCR assays, DNA was extracted from pure fungal cultures and from healthy wheat grain. *F. acuminatum* was not included in the qPCR assay due to limitations of time and resources. *F. graminearum* strain 38–49, *F. avenaceum* strain 29–31 and *F. poae* strain 30–21 were grown on PDA (BD) for one week prior to DNA extraction.

Mycelium was scraped using a spatula, placed in a 2-mL plastic tube (Eppendorf North America, Hauppauge, NY, USA) and freeze-dried. After the addition of a steel bead for each tube, fungal tissues were finely ground (1 min) by vortex. DNA was extracted following the protocols described by Covarelli et al. (2015a).

Uncontaminated grain of cv. Dyna-Gro Shirley was finely ground in a laboratory blender and DNA was extracted by the method described by Parry and Nicholson (1996) with some modifications. In brief, 10 mL of extraction buffer (hexadecyltrimethyl-ammonium bromide 8 g/L, *N*-lauroylsarcosine sodium salt 10 g/L, sorbitol 25 g/L, ethylenediamine-tetraacetic acid disodium salt dehydrate 8 g/L, polyvinylpolypyrrolidone 10 g/L, sodium chloride 87.6 g/L) (all from Sigma Aldrich), pre-heated at 65 °C, and 20 μL of RNAse A (20 mg/mL, Thermo Fisher Scientific, Waltham, MA, USA) were added to 2 g of ground winter wheat grains in a 50-mL plastic tube (Falcon, Thermo Fisher Scientific). After 1 min of vortexing, samples were placed for 1 h at 65 °C. After incubation, 2.5 mL of isoamyl alcohol-chloroform solution (1:24 v/v) (Sigma Aldrich) and 3.5 mL of potassium acetate (5 mol/L, Sigma-Aldrich) were added to the samples. After 30 s of vortex and incubation at −20 °C for 30 min, samples were centrifuged (1157 × *g*, 15 min, 5 °C) by Allegra X-30R centrifuge (Beckman Coulter). The upper phase (600 μL) was mixed in a solution of 600 μL of isopropanol and 60 μL of sodium acetate (0.1 mol/L) (Sigma Aldrich). After centrifugation (12,470 × *g* for 15 min) by centrifuge 5-42-4 (Eppendorf), DNA was washed twice in 70% ethanol (Sigma Aldrich) and resuspended in 75 μL of DNase free sterile water (Thermo Fisher Scientific). The isolated DNA samples were left overnight at 4 °C to aid resuspension and then stored at −20 °C.

The concentration of extracted DNA was measured by Qubit® 2.0 fluorimeter (Invitrogen, Life Technologies, Thermo Fisher Scientific) using a Qubit® dsDNA BR assay kit (molecular probes, Life Technologies, Thermo Fisher Scientific) and following the manufacturer's instructions.

Dilution series from 0.05 pg to 50 ng of three fungal strains DNA and from 5 pg to 50 ng of soft red winter wheat DNA, with a serial dilution factor of 10, were produced to set up standard curves that were processed in each q-PCR assay. Two replicates of each standard were used in each assay. Standard curves were generated by plotting the logarithmic values of known DNA quantities versus the corresponding cycle threshold (Ct) values. For each standard curve, from the average Ct of each dilution, the line equation ($y = mx + q$) was calculated as well as the R^2 value and reaction efficiency ($10^{(-1/m)}$). The limit of detection (LOD) of fungal biomass was 0.05 pg.

Total DNA from the milled samples (as previously noted, each sample consisted of the bulked 7 or 10 spikes per trial for each fungal species ∗ inoculation timing combination), including wheat DNA and potentially fungal DNA, was isolated using the method previously described for the uncontaminated wheat grain. The concentration of extracted DNA was estimated as previously described and the concentration of each DNA sample was adjusted to 20 ng/μL. The q-PCR analysis was carried out using specific primers for the detection and quantification of the three *Fusarium* species, while *translation elongation factor 1-α* (*tef1α*) primers were used for the quantification of wheat DNA (supplementary Table S1).

To optimize q-PCR reaction conditions, annealing temperatures (from 55 °C to 65 °C) were adjusted experimentally. The q-PCR assays were carried out in a CFX96 Real-Time System (Bio-Rad, Hercules, California, USA). The q-PCR mixture was composed of a total reaction

volume of 12 µL, containing 2.5 µL of total DNA, 6 µL of 2X SsoFast EvaGreen Supermix (Bio-Rad), 1.5 µL of 2 µM of each primer and 0.5 µL of sterile DNase free water (Thermo Fisher Scientific). The program consisted of: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 15 s and the specific annealing temperature of each primer (supplementary Table S1) for 1 min, heating at 95 °C for 10 s, cooling at 60 °C and finally an increase to 95 °C at 0.5 °C every 5 s with the measurement of fluorescence. A dissociation curve was included at the end of the q-PCR program to monitor the presence of potential primer-dimers and non-specific amplification products. Two analytical replicates of each sample were used in each assay. The fungal biomass in the soft red winter wheat grain was expressed as the ratio of the fungal DNA (pg) to the plant DNA (ng).

2.5. Detection and quantification of fungal secondary metabolites by LC-MS/MS

Five grams of each milled sample were extracted using 20 mL extraction solvent (acetonitrile-water-acetic acid, 79:20:1, v/v/v) followed by a 1 + 1 dilution using acetonitrile-water-acetic acid, (20:79:1, v/v/v) and direct injection of 5 µL diluted extract. LC-MS/MS screening of target fungal metabolites was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIon Spray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C_{18}-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA). The chromatographic method as well as chromatographic and mass spectrometric parameters are described in Malachova et al. (2014), but the method has in the meantime been expanded to cover > 650 metabolites.

ESI tandem mass spectrometry was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ± 27 s and ± 48 s in the positive and the negative mode, respectively. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of MON and 3-nitropropionic acid that exhibit only one fragment ion), which yielded 4.0 identification points according to commission decision (Commission Decision, 2002). In addition, the liquid chromatography retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% rel., respectively. Quantification was performed via external calibration using serial dilutions of a multi-analyte stock solution. Results were corrected for apparent recoveries obtained during re-validation of wheat for the extended set of analytes. The accuracy of the method is verified on a continuous basis by regular participation in proficiency testing schemes (De Girolamo et al., 2017; Malachova et al., 2014, 2015).

2.6. Statistical analysis

Disease severity data were analyzed using SAS software version 9.4 (SAS Institute Inc., Cary, NC). An analysis of variance was conducted using PROC GLIMMIX with *Fusarium* species and inoculation timing as independent variables. DS at the 21 dai assessment and the change in DS between 7 and 21 dai were each analyzed as dependent variables.

As indicated above, the experimental design included two experimental replicates (successive trials) with 7 or 10 independent biological replicates (spikes/plants) per trial. Within a trial, the design utilized a completely randomized factorial design, in which *Fusarium* species and inoculation timing were fixed-effect factors. Plants were randomly assigned to species ∗ inoculation timing treatments within each trial. The residual variance consisted of the within-group plant-to-plant variability averaged across treatment combinations. For the dependent response variable of DS at 21 dai, the residual variances for the first and second trial were 0.072 and 0.025, respectively, and since the resulting F-ratio was < 3 (2.84), the trials were subjected to a combined ANOVA.

The statistical model utilized a randomized complete block factorial design with two blocks, (the two trials). The additive linear model included as fixed effects the main effects of species, inoculation timing and their interaction. Random effects were block and block ∗ species ∗ inoculation timing, with the second term being the experimental error. The residual variance consisted of the pooled plant-to-plant variation over the two trials. There were four missing values in the first trial and one in the second trial.

Data analysis was performed using SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina). The GLIMMIX procedure was used to estimate model parameters, the least squares means, corresponding means comparisons, and tests of hypotheses. The Kenward-Roger option in the MODEL statement (ddfm = kr) was used to correct for the presence of missing values in the estimation of denominator degrees of freedom for the F values, when testing the null hypothesis of no fixed effect, and the estimation of standard errors of the predicted (marginal) means.

Fungal biomass and secondary metabolites data were analyzed using a similar model in PROC GLIMMIX, with pg of fungal DNA or µg/kg of the individual metabolites as the dependent variables. For fungal biomass, data were analyzed and means were separated using a log transformation where indicated to reduce heteroscedasticity, although untransformed values are shown in tables and figures for ease of interpretation. For secondary metabolites, where types of metabolites varied by species, data on each metabolite were analyzed within a species, using a log-transformation where plots of residuals vs. predicted values indicated improved homoscedasticity. For biomass and metabolites, means were separated using the Tukey-Kramer multiple comparison adjustment.

Finally, relationships among disease parameters were analyzed using linear regression with log-transformation where indicated.

3. Results

3.1. FHB disease severity in spikes

The species differed considerably in the FHB severity they caused. At all inoculation timings, the final (21 dai) mean head blight severity tended to be *F. graminearum* > *F. avenaceum* > *F. acuminatum* = *F. poae* (Fig. 1). *F. graminearum* caused final mean head blight severity of nearly 100% at all inoculation timings, which was higher than the other three species ($P \leq 0.04$) except when it was not different from *F. avenaceum* at 6 daa ($P = 0.55$). In turn, *F. avenaceum* was significantly higher than *F. acuminatum* and *F. poae* except at 9 daa, when those three species did not differ significantly ($P \geq 0.44$). While, *F. acuminatum* caused slightly greater severity than *F. poae* at all inoculation timings on a numerical basis, the difference was never significantly different ($P \geq 0.63$).

Considered individually, the species also differed as to the influence of inoculation timing on the disease severity they caused (Fig. 1). *F. graminearum* was unaffected by inoculation timing. *F. avenaceum* caused the most disease after inoculation at 6 daa, while for *F. acuminatum* it was at 6 and 9 daa, and for *F. poae* at 9 daa. Thus, the weaker pathogens caused greater disease severity at the later inoculation timings.

The three DS assessments, occurring at one-week intervals, allowed for the differentiation of inoculation-timing effects due to the greater number of extruded anthers at 9 daa vs. effects due to greater spread within the spikes (supplementary Fig. S1). In other words, within a *Fusarium* species, if later infection timings resulted in greater 7 dai severity, such differences would be too early to be explained by spread within the spike and instead would reflect a larger number of infections

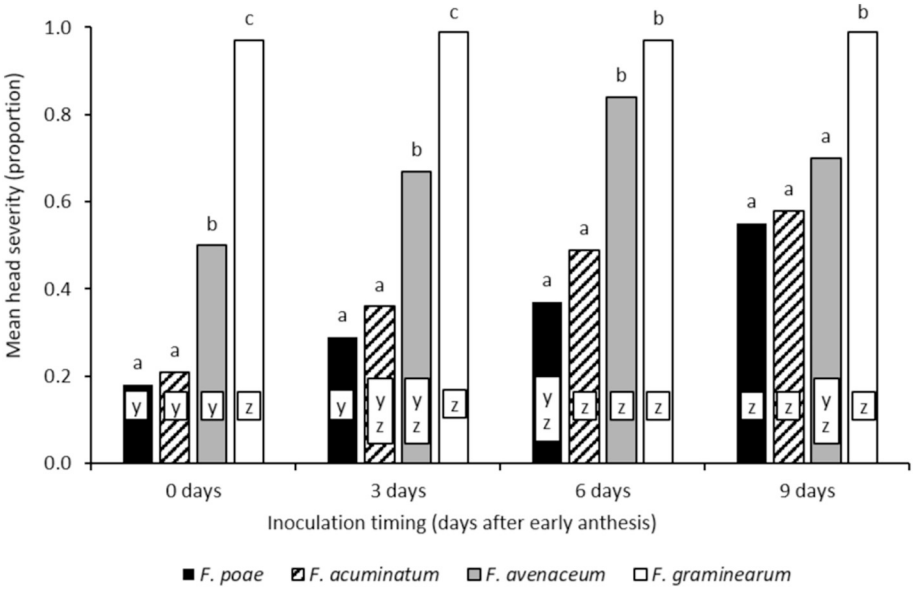

Fig. 1. Mean head blight severity at 21 days post-inoculation in wheat spikes inoculated in the greenhouse at 0, 3, 6 or 9 days after early anthesis with one strain each of four *Fusarium* species. Within an inoculation timing (a–c) or a *Fusarium* species (y–z), means with the same letter are not significantly different at $P \leq 0.05$ based on the Tukey-Kramer adjustment for multiple comparisons. Values are means of two successive trials with 7 and 10 spikes per strain * inoculation timing treatment, respectively.

due to the greater number of anthers extruded as flowering progressed, assuming that anthers remain hospitable to infection once extruded. In fact, at 7 dai, none of the species had significant differences in DS according to when they were inoculated ($P \geq 0.17$). In other words, the greater number of anthers extruded at 9 daa than at 0 daa did not lead to greater expression of symptoms at 7 days following the inoculation.

Inoculation timing did have different effects on the change in disease severity between 7 dai and 21 dai, depending on the pathogen species (Table 1). For the two weakest pathogens, *F. poae* and *F. acuminatum*, inoculation timing had a significant impact ($P \leq 0.04$), with the 9 daa inoculation resulting in greater increase from 7 to 21 dai than the 0 daa inoculation. As there was no significant difference in DS at 7 dai regardless of inoculation timing, it is reasonable to conclude that this greater 7 to 21 dai DS change for the later inoculation timings was due to greater spread within the *F. poae* and *F. acuminatum*-infected spikes following the later inoculations. By contrast, there was no significant effect of infection timing on the 7 to 21 dai DS change for *F. avenaceum* or *F. graminearum* (Table 1). This lack of a timing effect on the spread within spikes of the more aggressive pathogens indicated they could colonize well at all growth stages where infection was tested, whereas for the weaker pathogens, colonization was favored by the later infection timings.

Table 1
Increase in Fusarium head blight severity between assessments at 7 and 21 days after inoculation at four timings (0, 3, 6 and 9 days after anthesis) with four *Fusarium* strains in the greenhouse.

Inoculation timing (days after anthesis)	Mean spikelet severity (proportion)[z]				
	F. poae	*F. acuminatum*	*F. avenaceum*	*F. graminearum*	Mean
0 days	0.14a	0.16a	0.30a	0.33a	0.23a
3 days	0.19a	0.28ab	0.43a	0.24a	0.29b
6 days	0.30ab	0.35ab	0.45a	0.15a	0.31ab
9 days	0.50b	0.50b	0.45a	0.27a	0.43b
Mean	0.28a	0.32a	0.41a	0.25a	

Means separation applies within a column or, in the case of the overall species means, within the row; means followed by the same letter are not significantly different ($P \geq 0.05$) based on the Tukey-Kramer adjustment for multiple comparisons.

[z] Values are means from two trials in time, with 7 and 10 replicate plants per species * inoculation timing combination in the first and second trials, respectively.

This was related to the early advantage of *F. graminearum*: by 7 dai, that species had already caused ≥65% DS at all infection timings, compared to 20–40% DS for *F. avenaceum* and 4–15% for the two weaker species (supplementary Fig. S1).

3.2. Fungal biomass accumulation in grain

R^2 values calculated from the linear equations of the four standard curves were 0.99 each for soft wheat, *F. graminearum*, *F. avenaceum* and *F. poae*. Reaction efficiencies obtained from the linear equations of the four standard curves were 98% in all four cases. The dissociation curve analysis showed specific amplification products in the presence of pure fungal DNA (standard curves) and in the presence of DNA of the three *Fusarium* species analyzed (samples). No target amplification was detected in negative controls including those lacking DNA of the three *Fusarium* species. Therefore, the Ct values used to quantify fungal biomass were those for which dissociation curve analysis showed the presence of specific amplification products.

All three species (*F. poae*, *F. avenaceum* and *F. graminearum*) analyzed by q-PCR were detectable through the presence of their biomass (pg of fungal DNA/ng of plant DNA) in the grain harvested at physiological maturity, and biomass accumulation of the three species differed. In general, fungal biomass was *F. graminearum* > *F. avenaceum* > *F. poae* (Fig. 2). *F. graminearum* biomass was always higher ($P \leq 0.001$) than that of the other two species, and *F. avenaceum* biomass was higher than that of *F. poae* ($P = 0.01$) except at 9 daa ($P = 0.48$). Proportionally, the greatest differences in biomass accumulation between *F. graminearum* and the other two species were detected at 3 daa and 6 daa inoculation timings.

F. graminearum biomass was strongly affected by inoculation timing (Fig. 2). The biomass ranking was 3 daa ≥ 6 daa ≥ 0 daa = 9 daa. The accumulation of *F. graminearum* fungal biomass was higher after inoculation at 3 daa than from the other inoculation timings except after inoculation at 6 daa ($P = 0.68$). From this inoculation timing (6 daa), biomass of *F. graminearum* was higher than from 0 daa and 9 daa, but the difference was not significantly different ($P = 0.30$ and $P = 0.25$, respectively). Thus, the highest rate of grain infection as measured by fungal biomass abundance was reached by *F. graminearum* at the intermediate inoculation timings (3 daa and 6 daa) rather than at the early (0 daa) and late (9 daa) timings.

F. avenaceum showed a similar pattern as *F. graminearum* following the four inoculation timings. The greatest fungal biomass was recovered after inoculation at 3 daa and 6 daa, which were not significantly

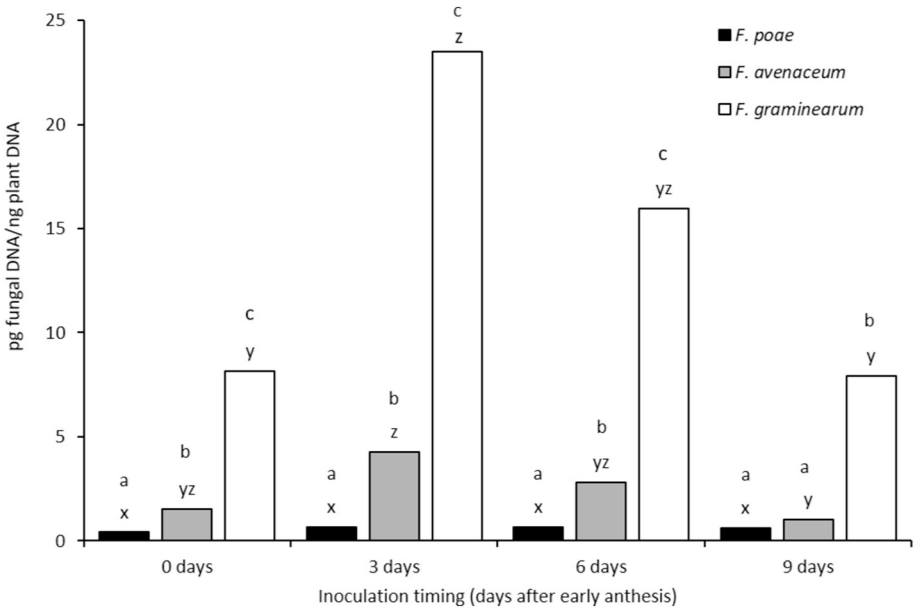

Fig. 2. Mean fungal biomass (as detected by real time quantitative PCR) of *F. poae*, *F. avenaceum* and *F. graminearum* strains in winter wheat kernels harvested at physiological maturity after spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis. Values are means of two biological replicates. Within an inoculation timing (a–c) or species (x–z), means with the same letter are not different at $P \leq 0.05$ based on the Tukey-Kramer adjustment for multiple comparisons.

different ($P = 0.58$). Biomass from the 3 daa inoculation was nearly (but not significantly) greater than that from the 0 daa inoculation, and the 6 daa biomass was nearly greater than the 9 daa biomass ($P = 0.06$ in both cases). *F. poae* exhibited a different profile of biomass accumulation in the grain with respect to inoculation timing; the biomass accumulated after the four inoculation timings did not differ significantly ($P \geq 0.60$).

Thus, colonization of the weaker pathogens was proportionally less influenced by different inoculation timings than that of the aggressive pathogen. Specifically, the gradient of inoculation timing influence tended to be *F. graminearum* ≫ *F. avenaceum* > *F. poae*.

3.3. Secondary metabolites in grain

All four inoculated species (*F. graminearum*, *F. avenaceum*, *F. acuminatum* and *F. poae*) biosynthesized a specific range of secondary metabolites detected by LC-MS/MS in grain harvested at physiological maturity. The profile of secondary metabolites differed depending on the *Fusarium* species.

The *F. graminearum* secondary metabolites that had accumulated after spike inoculation in the greenhouse at 0, 3, 6 or 9 daa are reported in detail in supplementary Table S2. In general, secondary metabolite accumulation tended to be higher from inoculations at 3 and 6 daa than at 0 and 9 daa (Fig. 3A–B, Supplementary Table S2). Among the secondary metabolites, DON, 15AcDON and 5-hydroxyculmorin were present in the highest concentrations (supplementary Table S2). The accumulation of DON was higher after inoculation at 3 daa than from the other inoculation timings ($P \leq 0.02$) except after inoculation at 6 daa ($P = 0.25$) (Fig. 3A). The DON:15AcDON ratio ranged from 4.1 (9 daa infections) to about 6 (3 daa and 6 daa) to 13.7 (0 daa). The accumulation of 15AcDON followed a similar trend, with a higher level detected from inoculation at 3 daa than from 0 daa ($P \leq 0.04$), while 6 daa and 9 daa levels were intermediate (Fig. 3B). In summary, a higher rate of DON and 15AcDON accumulation in the grain was reached at the intermediate *F. graminearum* inoculation timings, in particular at 3 daa, rather than at the early or late inoculation timings. As for the other *F. graminearum* secondary metabolites, a statistically significant peak of accumulation from 3-daa inoculation, in comparison to the earliest or latest inoculation timings, was reached by 15-hydroxyculmoron, 5-hydroxyculmorin, and chrysogin (supplementary Table S2).

The *F. avenaceum* secondary metabolites that accumulated following

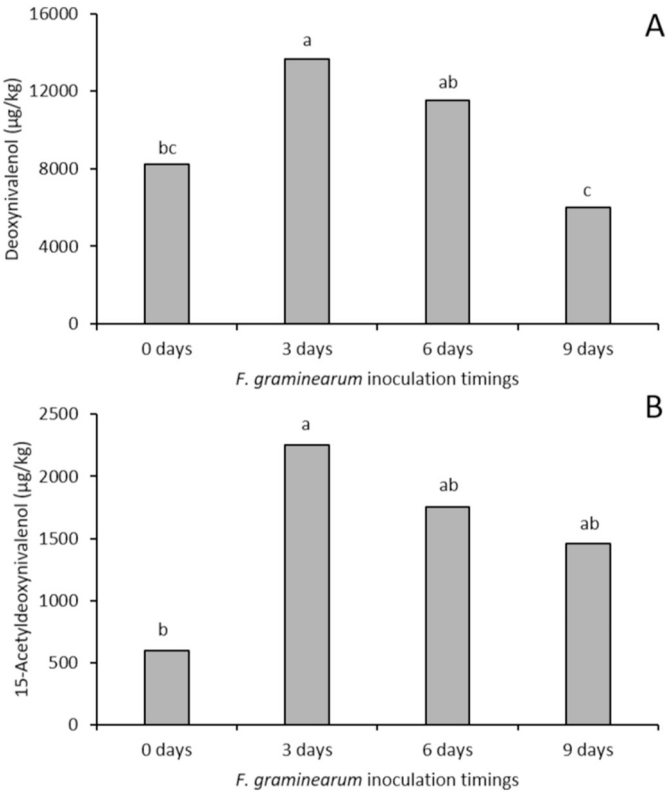

Fig. 3. Mean concentrations of deoxynivalenol (A) and 15-acetyldeoxynivalenol (B) (µg/kg) in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis with *F. graminearum* and harvest at physiological maturity. Values are means of two biological replicates. Means topped by the same letter are not different at $P \leq 0.05$ based on the Tukey-Kramer adjustment for multiple comparisons.

the different inoculation timings were summarized in supplementary Table S3. With the exception of ENB, ENB1 and butenolide, the secondary metabolites produced by *F. avenaceum* tended to be higher at the intermediate inoculation timings (3 daa and 6 daa) than at the early and late ones (0 daa and 9 daa). However, the differences among inoculation timings were never statistically significant ($P \geq 0.05$) for any secondary

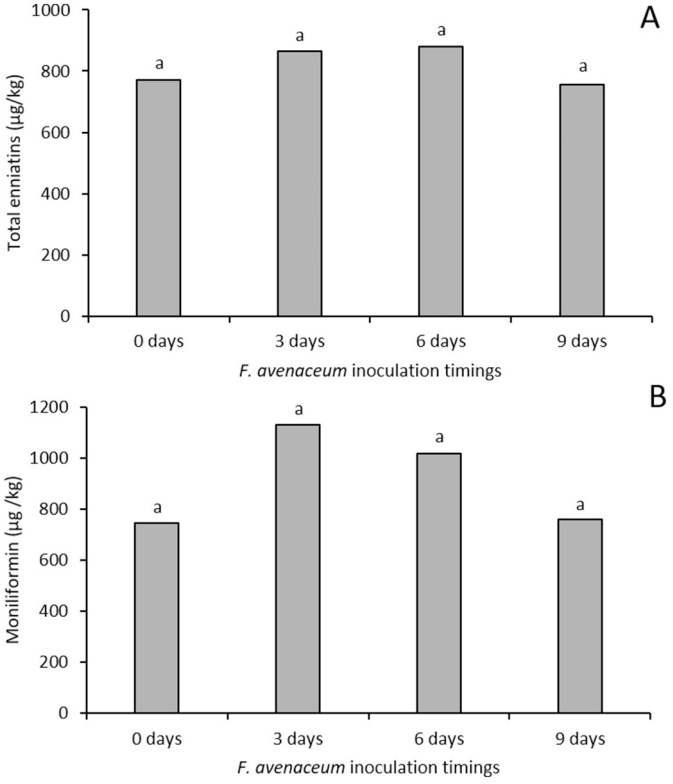

Fig. 4. Mean concentrations of total enniatins (A) and moniliformin (B) (µg/kg) in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis with *F. avenaceum* and harvest at physiological maturity. Values are means of two biological replicates. Means topped by the same letter are not different at $P \leq 0.05$ based on the Tukey-Kramer adjustment for multiple comparisons.

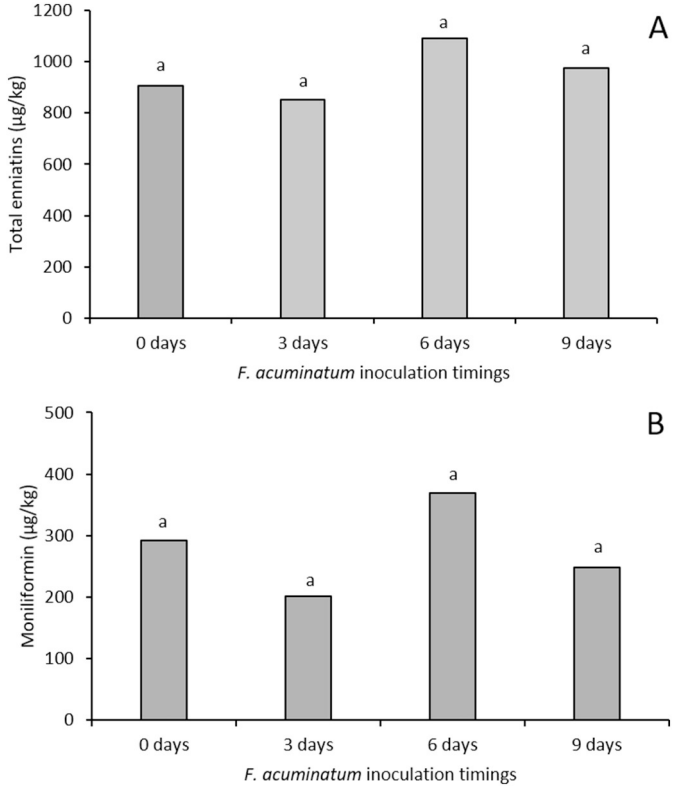

Fig. 5. Mean concentrations of total enniatins (A) and moniliformin (B) (µg/kg) in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis by *F. acuminatum* and harvest at physiological maturity. Values are means of two biological replicates. Means topped by the same letter are not different at $P \leq 0.05$ based on the Tukey-Kramer adjustment for multiple comparisons.

metabolites (supplementary Table S3). Total ENs (calculated by the sum of all six analogues analyzed) and MON were the secondary metabolites present in highest concentration at all four inoculation timings with *F. avenaceum*. EN analogue concentrations displayed the following gradient: ENB1 > ENB2 > ENA1 > ENB > ENA > ENB3. Total ENs and MON, despite their numerically higher levels after 3 daa and 6 daa, did not differ significantly by inoculation timing ($P \geq 0.38$) (Fig. 4A–B). In these experimental conditions, accumulation of these two secondary metabolites was not particularly affected by infection timing during anthesis.

The *F. acuminatum* secondary metabolites that accumulated following the different spike inoculation timings are summarized in supplementary Table S4. A general increase at the 6 daa timing for ENA, ENA1, ENB1, ENB2, ENB3, total ENs, MON, chlamydospordiol, chlamydosporol, chrysogin and aminodimethyloctadecanol was observed. However, the differences among inoculation timings were never significant ($P \geq 0.17$) for any secondary metabolites (supplementary Table S4). Similar to *F. avenaceum*, total ENs (calculated as the sum of all six analogues analyzed) were the secondary metabolites with highest concentrations after inoculation with *F. acuminatum*, with MON the most concentrated of the other metabolites. However, ENs analogues accumulated with a different gradient in comparison to *F. avenaceum*: ENA1 > ENB1 > ENA > ENB > ENB2 > ENB3. Total ENs and MON, despite their higher levels after 6 daa, did not differ significantly by inoculation timing ($P \geq 0.37$) (Fig. 5A–B); as with *F. avenaceum*, the accumulation of these two secondary metabolites was not particularly affected by this parameter.

Comparing *F. acuminatum* and *F. avenaceum* for their principal metabolite types, the levels of total ENs across inoculation timings as well as within each inoculation timing were not significantly different ($P = 0.97$ and $P \geq 0.98$ respectively). However, MON concentration from *F. avenaceum* was significantly higher across inoculation timings ($P = 0.002$) than that from *F. acuminatum*. These differences were significant after the intermediate inoculation timings (3 daa and 6 daa) ($P \leq 0.02$) but not after the early and late inoculation timings (0 daa and 9 daa) ($P \geq 0.07$).

The *F. poae* secondary metabolites that accumulated following the different spike inoculation timings are summarized in supplementary Table S5. Similar to *F. acuminatum*, a general increase at the 6 daa timing for all secondary metabolites was observed with the exception of all EN analogues, which tended to be higher from early-intermediate inoculation timings (0 daa and 3 daa). However, the differences among inoculation timings were never significant ($P \geq 0.09$) for any secondary metabolites (supplementary table S4). NIV, total ENs (calculated as the sum of all six analogues analyzed) and BEA were the most concentrated secondary metabolites produced by *F. poae*. EN analogues accumulated with a different gradient in comparison to *F. avenaceum* and *F. acuminatum*: ENB > ENB1 > ENB2 > ENA1 > ENA > ENB3. Despite numerically higher levels after 6 daa (NIV and BEA) or after 0 daa and 3 daa (total ENs), none of these inoculation timings were significantly different ($P \geq 0.39$) (Fig. 6A–C) thus, with *F. poae*, infection timing did not affect accumulation of these three secondary metabolites.

Overall, concentrations of *F. poae* secondary metabolites were low compared to those produced by the other species. The levels of total ENs after inoculation with *F. poae* across inoculation timings and within each timing was significantly lower ($P \leq 1 * 10^{-4}$ and $P \leq 0.007$ respectively) than those recovered after inoculation with *F. avenaceum* or *F. acuminatum*. However, BEA after inoculation with *F. poae* was significantly higher than that from *F. acuminatum*, both across timings and within each one ($P \leq 1 * 10^{-4}$). The absence of T-2 and HT-2 toxins in

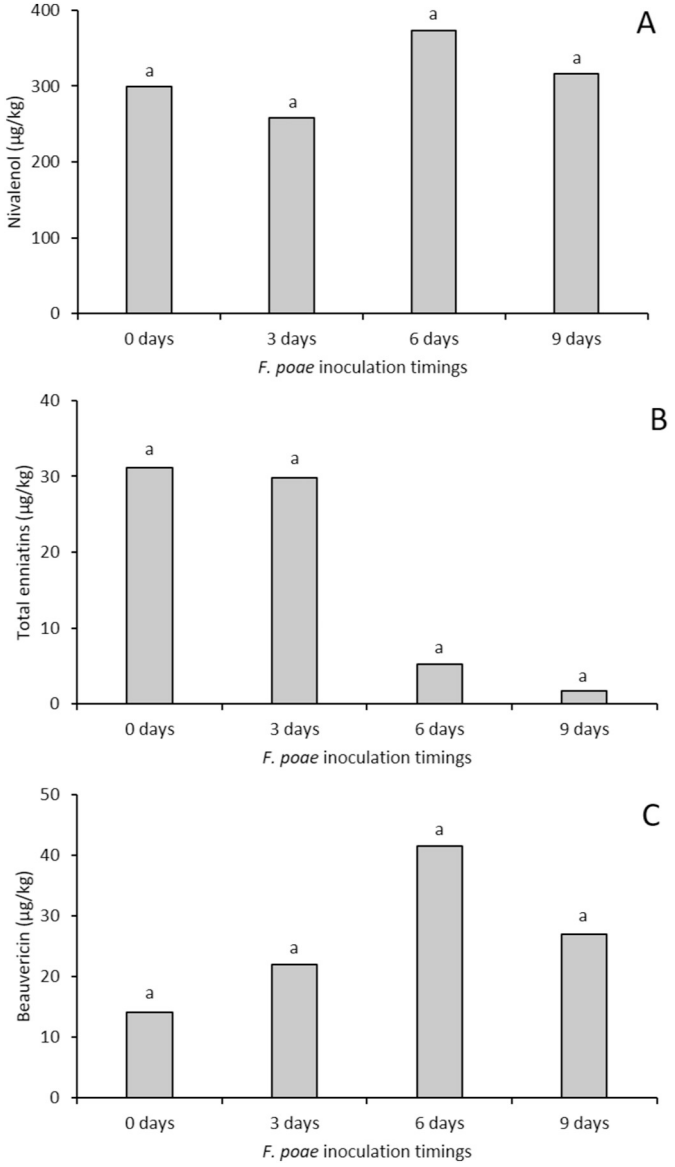

Fig. 6. Mean concentrations of nivalenol (A), total enniatins (B) and beauvericin (C) (µg/kg) in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis by *F. poae* and harvest at physiological maturity. Values are means of two biological replicates. Means topped by the same letter are not different at $P \leq 0.05$ based on the Tukey-Kramer adjustment for multiple comparisons.

the grain after inoculation with *F. poae* showed that the strain used in this experiment likely produces little or none of these two mycotoxins.

3.4. Relationships among FHB parameters

Taking the three species (*F. graminearum*, *F. avenaceum* and *F. poae*) together, fungal biomass was positively related to DS at 21 dai (adj. $R^2 = 0.76$, $P \leq 1 * 10^{-4}$; Fig. 7). However, none of the individual strain relationships between DS at 21 dai and biomass were significant at better than $P = 0.09$ (Fig. 7; supplementary Fig. S2; supplementary Fig. S3). The overall significant association was due principally to the two weaker pathogens, *F. avenaceum* and *F. poae*. In fact, for *F. graminearum*, DS was nearly 100% at 21 dai for all inoculation timings (Fig. 1), while biomass varied significantly across timings (Fig. 2); thus, the relationship of DS at 21 dai and biomass for that species was nonsignificant ($P = 0.70$; Fig. 7). A stronger relationship between DS and biomass was observed for *F. graminearum* at 7 dai ($P = 0.09$; supplementary Fig. S2)

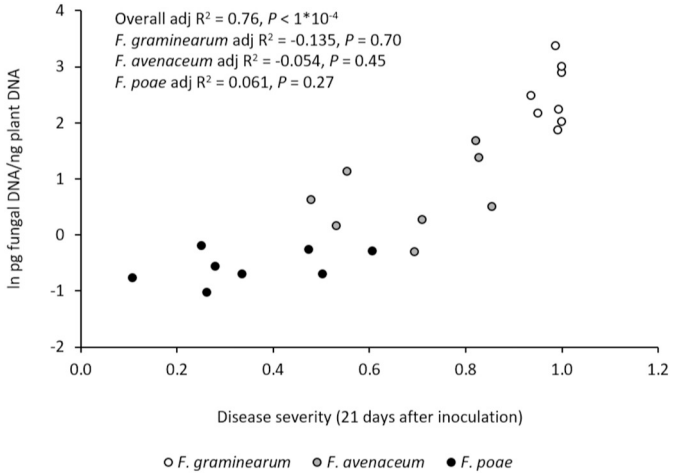

Fig. 7. Correlation between disease severity (21 days after inoculation) and colonization (pg of fungal DNA/ng plant DNA) of *F. graminearum*, *F. avenaceum* or *F. poae* in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis. The data of fungal biomass are natural log-transformed. Both replicates are shown separately.

followed by the relationship at 14 dai ($P = 0.50$; supplementary Fig. S3). The DS at 21 dai-DON relationship for *F. graminearum* was similarly nonsignificant ($P = 0.74$).

Conversely, for *F. graminearum*, the relationship between biomass and DON production was strongly positive (adj. $R^2 = 0.87$, $P = 4 * 10^{-4}$; Fig. 8) and the same was true for biomass and 15AcDON (adj. $R^2 = 0.69$, $P = 0.007$). With respect to one of the two major depsipeptide producers, *F. avenaceum*, there was also a strong positive association between biomass and both total ENs (adj. $R^2 = 0.63$, $P = 0.01$) and the sum of ENs and MON (adj. $R^2 = 0.68$, $P = 0.007$; Fig. 9). The relationship between DS (7, 14 and 21 dai) and ENs, MON, or their sum was always non-significant; it was nearly significant for 21-dai DS (adj. $R^2 = 0.38$–0.40, $P = 0.053$–0.06), but not for the earlier assessments ($P \geq 0.23$). For both *F. graminearum* and *F. avenaceum*, the ratio of metabolite production to biomass was higher for the early and late inoculation timings and lower for 3- and 6-daa inoculations (Figs. 8 and 9). For *F. poae*, the accumulation of total ENs, BEA and NIV showed an absence of correlation both with biomass in the grain and with DS ($P \geq 0.13$).

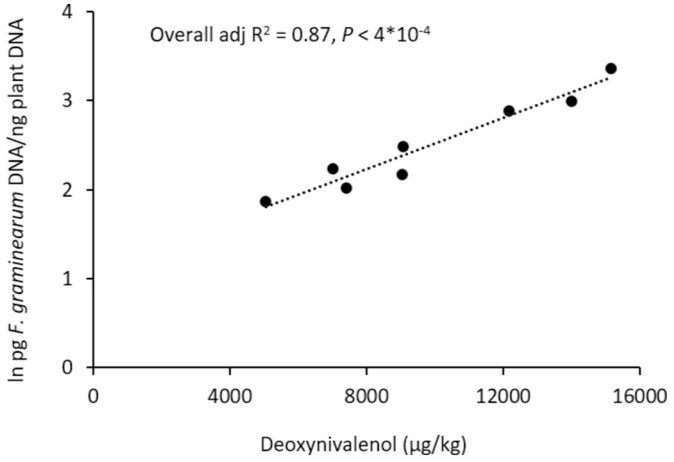

Fig. 8. Relationship between deoxynivalenol (DON, µg/kg) and colonization (pg of fungal DNA/ng plant DNA) of *F. graminearum* in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis. Fungal biomass values are natural log-transformed. Both replicates are shown separately. Averaging across replicates, DON:biomass ratios were 1007, 599, 730, and 764 for the four inoculation timings, respectively.

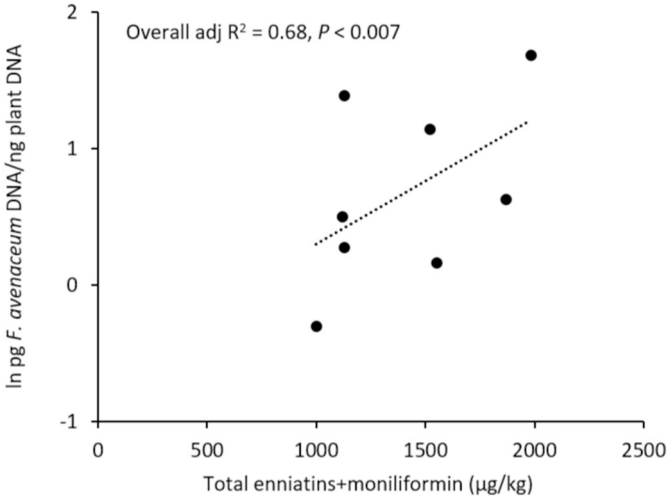

Fig. 9. Relationship between total enniatins + moniliformin (ENs + MON, µg/kg) and colonization (pg of fungal DNA/ng plant DNA) of *F. avenaceum* in winter wheat kernels following spike inoculation in the greenhouse at 0, 3, 6, or 9 days after early anthesis. Fungal biomass values are natural log-transformed. Both replicates are shown separately. Averaging across replicates, ENs + MON:biomass ratios were 1072, 475, 787, and 1567 for the four inoculation timings, respectively.

4. Discussion

In this controlled-environment study, the *F. graminearum* strain strongly outperformed the other three species in FHB-causing ability. *F. avenaceum* was intermediate, and *F. acuminatum* and *F. poae* were the less aggressive species. Although only a single strain of each species was used, this ranking is consistent with relative frequencies of the various pathogens in Canadian field samples (Tittlemeier et al., 2013) and Europe (Lindblad et al., 2013).

F. poae and *F. acuminatum* produced greater final (21 dai) DS if they had only late infection opportunities (6 and 9 daa), while *F. avenaceum* peaked in FHB-causing ability when inoculated at 6 daa. By contrast, *F. graminearum* caused equal levels of symptoms with all studied timings, although it produced more biomass and as a result more DON when infecting at 3 daa. This suggests that the timing of favorable weather in relationship to wheat anthesis may affect the success of the weaker, non-*graminearum* species individually, and also the balance among the *Fusarium* species when more than one is present. Although this was not a competition study, it seems possible that when *F. graminearum* is present along with one or more of the other species, it will out-perform them when conditions result in spore dispersal earlier in the anthesis period. If conditions are first conducive for spore release later in anthesis, the weaker species may compete better against *F. graminearum*.

The degree of 7- to 21-dai spread within spikes was not associated with infection timing for *F. graminearum* and *F. avenaceum*, but was greater at the latest infection timing for *F. poae* and *F. acuminatum*. The data suggest that the improved performance following late infections that was observed for *F. poae* and *F. acuminatum* was particularly due, not to the number of anthers extruded, but to greater spread within the spikes.

As for the physiological basis of the observed differences, the advantage of *F. graminearum* over other species at the earlier infection timings may be due to greater infection efficiency and/or more rapid and extensive spread within spikes. DON is a pathogenicity factor that facilitates spread within spikes, whereas the role of MON and ENs in infection and spread within the spike is unknown (Bai et al., 2002; Cole et al., 1973; Jansen et al., 2005; Wakulinski, 1989). It is possible that DON produced by *F. graminearum* gives equivalent advantage in spreading within spikes at all the tested timings, whereas the ENs and MON confer greater advantage later in the process. For example, Tittlemeier et al. (2013) suggest that MON and ENs may be upregulated when *Fusarium* has used up nutrients from the endosperm.

In this study, we found that biomass of *F. graminearum* in wheat grain was higher than that of the weaker pathogens (*F. avenaceum* and *F. poae*), which agreed with other reports on the relative aggressiveness of the tested species (Brennan et al., 2003; Siou et al., 2014; Stenglein, 2009). However, the greatest differences in biomass accumulation were observed after inoculation at 3 daa and 6 daa. At early and late anthesis (within the time window evaluated in this experiment), the differences in colonization between the more aggressive and weaker species were reduced. As the experimental conditions used in this research were optimal for FHB development with respect to temperature and humidity, we conclude that infection timing likely plays a role in the different incidence of FHB causal agents. Timing of favorable weather in relationship to anthesis may shift the balance among different *Fusarium* species within the FHB complex.

Anthesis stage affected biomass accumulation of *F. graminearum* in kernels. In particular, our results suggest that within the period of maximum receptivity of wheat to *F. graminearum* infection, which is thought to be until around 10 days after mid anthesis (Cowger and Arellano, 2010), there could be a sub-period (including 3 to 6 days after early anthesis) during which susceptibility is at its maximum. However, the use of a single strain per species does not allow a general conclusion to be drawn at the species level. At the same time, inoculation timing had a more moderate effect on biomass accumulation of the weaker species *F. avenaceum* and no effect on *F. poae*. The absence of an influence of inoculation timing on *F. poae* fungal biomass was also described by Siou et al. (2014).

Secondary metabolites biosynthesized by the aggressive species *F. graminearum* were more affected by infection timing than those produced by the weaker species *F. avenaceum*, *F. acuminatum* and *F. poae*. A higher level of all secondary metabolites was observed in the grain when *F. graminearum* infected the spikes at 3 daa, and this was particularly true for DON and 15AcDON, the mycotoxins at highest concentration. Both compounds were higher after inoculation at 3 daa than at 0 daa, indicating that a difference of only 3 days was sufficient to significantly affect DON and/or 15AcDON content in the grain at maturity. This suggests that day-to-day weather variation may substantially change DON and 15AcDON accumulation in grain when the variation occurs at the right time interval with respect to anthesis.

The concentration of DON and 15AcDON mycotoxins showed a higher correlation with *F. graminearum* biomass in the grain than with DS on spikes. In addition, our data also showed that DS at 7 dai was a more reliable estimator of DON and 15AcDON in mature grain than DS at 14 or 21 dai. Thus, DS may be a poor estimator of DON and15AcDON accumulation at maturity, especially when conditions favor late infections over early ones.

Further, the correlation of toxin concentrations and biomass suggested that differences in DON and 15AcDON from different infection timings were due to colonization and not to differential stimulation of mycotoxin biosynthetic pathways. Indeed, while *F. graminearum* biomass and DON were highest from the 3-daa inoculations, the ratio of DON to biomass was lowest from that timing. The same was true for *F. avenaceum* with respect to total ENs. In other words, while 3-daa infections were maximally conducive to spread within the spike, they were the least efficient timing for metabolite production. If infections at different timings resulted in differential stimulation of mycotoxin biosynthesis, we were unable to see evidence of it.

After *F. graminearum* inoculation, DON accumulation in grain substantially exceeded 15AcDON. The DON:15AcDON ratio increased as infection timing became later; it would be necessary to use more *F. graminearum* isolates to determine if this is a broad effect on the relative abundance of the acetylated form.

Total ENs, MON, NIV and BEA were present in measurable amounts after inoculation with the weaker species at all inoculation timings.

This suggests that the secondary metabolites biosynthesized by the weaker species may occur as a consequence of infections at any point during the anthesis period. However, secondary metabolites produced by the weaker species (*F. avenaceum*, *F. acuminatum* and *F. poae*) were less affected by infection timing than those produced by *F. graminearum*. With the weaker species, a general (although non-significant) increase in key compounds at specific inoculation timings was noticed. This suggests that a more subtle influence of infection timing could occur in the field, and could be detectable with higher experimental power. Although *F. graminearum* produced higher levels of most secondary metabolites after the 3 daa inoculation, with the weaker species a slight shift later was observed: for *F. avenaceum*, from 3 or 6 daa inoculations; for *F. acuminatum* from 6 daa; and for *F. poae*, from 6 daa except for ENs, which were at very low levels. This was particularly the case with *F. avenaceum* and *F. acuminatum* for total ENs and MON and with *F. poae* for NIV and BEA. For this reason, we hypothesize that the secondary metabolites produced by these weaker FHB causal agents could be favored when environmental conditions become conducive a bit later relative to anthesis, while those produced by *F. graminearum* are relatively favored by slightly earlier conducive conditions.

At < 1100 μg/kg, total concentrations of the depsipeptides ENs and BEA in this study were on the low end of the range detected in field samples of wheat. For example, Canadian and Finnish wheat samples predominantly infected with *F. avenaceum* contained a mean of 3800 and 7200 μg/kg of depsipeptides, respectively (Logrieco et al., 2002; Tittlemeier et al., 2013). In our study, BEA was only produced in appreciable concentrations (14–42 μg/kg) by *F. poae* (Supplementary Table S4). For comparison, BEA was detected at concentrations < 10 μg/kg in field samples from Norway and Finland (Jestoi, 2008).

Among the other fungal secondary metabolites detected, the grain in our experiment contained a wide range of compounds that are important for the physiological functions of the fungal species, and may also be implicated in their interactions with plants and other microorganisms, as well as having a negative impact on consumers' health. For example, considering the compounds detected only after inoculation with *F. graminearum*, gibepyrone D is known for its nematocidal activity (Bogner et al., 2017); fusarin C can act like an estrogenic agonist and stimulates breast cancer cells in vitro (Sondergaard et al., 2011); culmorin, detected along with its derivatives such as 15-hydroxyculmorin, 5-hydroxyculmorin and 15-hydroxyculmoron, showed antifungal activity, phytotoxicity and enhanced DON toxicity to insects impacting both growth and mortality (McCormick et al., 2010); and sambucinol showed a potent synergism with DON on insects (Dowd et al., 1989).

With respect to the secondary metabolites recovered only after inoculation with *F. avenaceum* and *F. acuminatum*, antibiotic Y showed phytotoxic activity and inhibitory effects on bacterial growth (Golinski et al., 1996); aminodimethyloctadecanol, to our knowledge, has no particular reports in the scientific literature on its toxicity and/or biological activity; and chlamydosporol, detected along with its related metabolite chlamydospordiol, has shown toxicity to human cell lines and insects (Savard et al., 1990; Solfrizzo and Visconti, 1991).

Finally, considering the secondary metabolites detected after inoculation with all four species, aurofusarin is a pigment with antibiotic properties against both mycelial fungi and yeasts (Medentsev et al., 1993) but is also able to induce oxidative stress, cytotoxicity and genotoxicity in human colon cells (Jarolim et al., 2018); rubrofusarin is another pigment that acts as an intermediate in the aurofusarin biosynthetic pathway (Frandsen et al., 2006); butenolide can induce dysfunction of myocardial mitochondria (Wang et al., 2009); and chrysogin has no reports in the scientific literature about its activity. However, further research on many of the secondary metabolites detected in this study will be necessary to better understand their role in the above-mentioned interactions, as well as their impact on grain quality and consumer health.

Accumulation of total ENs and MON showed a higher correlation with *F. avenaceum* biomass in the grain than with symptoms. This suggest that DS may be a somewhat weak estimator of total ENs and MON accumulation at grain maturity after *F. avenaceum* infection, even if the relationship is stronger than with DON and *F. graminearum*. Despite higher symptoms due to greater spread after later infections, biomass of *F. poae* and *F. avenaceum* remained relatively low from all infection timings, just as their production of ENs and MON was fairly consistent across infection timings. The increased symptom development following later inoculations was not matched by increased biomass of these pathogens. At the same time, *F. avenaceum* biomass and ENs production were positively associated, which is logical. In contrast to what was observed for *F. graminearum*, the reliability of total ENs and MON estimation from symptoms increased with time post-inoculation. That is, disease severity at 21 dai was a more reliable estimator of total ENs and MON presence in the grains at maturity than disease severity at 7 dai and 14 dai.

Interestingly, in the aggressive species *F. graminearum*, symptom development was unrelated to biomass and DON production: symptoms were consistent across infection timings, based on severity at 21 dai, while biomass and DON were not. The discrepancy between the external and internal parameters was highest after early and late inoculation timings. As reported by Siou et al. (2014), visual assessment of disease on the spike appeared to be a poor estimator of the actual infection level in kernels for those early or late infections. These authors found that the correlation between visual symptoms and *F. graminearum* biomass was high for inoculations performed around anthesis (0 daa and 8 daa) but rather low or even non-significant for earlier or later inoculation dates. Our research, although it covered a more restricted time window during anthesis, led to a similar conclusion. This discrepancy could be due to several factors. One possibility is that externally visible symptoms primarily reflected fungal development in the glumes (Xu et al., 2008), while we only quantified fungal biomass in the kernels. Efficiency of kernel infection may have been the parameter most affected by the varying timings of inoculation with *F. graminearum*.

In summary, this study provided new information on FHB symptom occurrence, fungal biomass and fungal secondary metabolite accumulation as influenced by infection timing of the aggressive species *F. graminearum* in comparison with the relatively weak species *F. avenaceum*, *F. poae* and *F. acuminatum*. Infection timing appears to play a role in particular in *F. graminearum* fungal biomass, which was far more abundant than that of the other species, and in its secondary metabolite accumulation. However, secondary metabolites produced by weaker species could be relatively favored when environmental conditions become conducive later in the overall critical period, while those produced by *F. graminearum* are advantaged by slightly earlier conducive conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.10.014.

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