Research Article

Infection timing affects *Fusarium poae* colonization of bread wheat spikes and mycotoxin accumulation in the grain

Francesco Tini, Lorenzo Covarelli, Christina Cowger, Michael Sulyok, Paolo Benincasa and Giovanni Beccaria

Abstract

**BACKGROUND:** *Fusarium poae* is one of the most common *Fusarium* head blight (FHB) causal agents in wheat. This species can biosynthesize a wide range of mycotoxins, in particular nivalenol (NIV). In FHB epidemiology, infection timing is important for disease occurrence, kernel development, symptom appearance and mycotoxin accumulation in grain. The present study explored, both in a controlled environment and in a 2-year field plot experiment in Central Italy, the influence of five infection timings (from beginning of flowering to medium milk growth stage) on *F. poae* colonization and mycotoxin accumulation in bread wheat spikes (spring cv. A416 and winter cv. Ambrogio).

**RESULTS:** Both climate chamber and field experiments showed that early infection timings (from beginning of flowering to full flowering) especially favoured *F. poae* colonization and accumulation of its mycotoxins (particularly NIV) in grain. By contrast, later infection timings (watery ripe and medium milk) reduced *F. poae* development and mycotoxin levels. The time window of host susceptibility in the field was shorter than that observed under controlled conditions. Symptom expression in kernels also differed among infection timings. In general, *F. poae* biomass was higher in the chaff than in the grain.

**CONCLUSION:** These results enhance knowledge of a common member of the FHB complex worldwide, and could be useful in forecasting the risk of *F. poae* infection and mycotoxin contamination.

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**Keywords:** *Fusarium poae*; mycotoxins; nivalenol; wheat; cereals; infection timing

**INTRODUCTION**

*Fusarium* head blight (FHB) is one of the most widespread and destructive diseases of wheat, causing serious damage because of the reduced quality and quantity of grain.  

This disease is caused by many species belonging to the genus *Fusarium,* which can biosynthesize a wide range of mycotoxins, comprising secondary metabolites with toxic effects on human and animal health. Among the different *Fusarium* species able to cause FHB, *Fusarium graminearum* is considered to be the most common globally.  

However, recently, other species such as *Fusarium poae* and *Fusarium avenaceum* have significantly increased in incidence, becoming the most frequent species within the FHB complex in some cereal-producing regions in some years.  

Generally, *F. poae* is considered a ‘weak’ pathogen compared to *F. graminearum,* although the real extent of its presence may have been underestimated because of the difficulty in detecting visual symptoms.  

*Fusarium poae* infections in cereals are particularly dangerous because of the ability of this pathogen to produce a suite of mycotoxins. *Fusarium poae* is known as one of the most important nivalenol (NIV) producers, a mycotoxin belonging to the chemical family of type B trichothecenes. Based on current knowledge, among *F. poae* secondary metabolites, NIV could be considered to be the most dangerous to human and animal health because of its frequency and toxicity.  

Although not as prevalent as the best-known type B trichothecene, deoxynivalenol (DON), NIV has shown higher acute toxicity to animals than DON, as well as...
as the highest in vitro immunosuppressive effects on human cells. For these reasons, NIV creates concerns about food safety, although in vivo information for assessing human health risks remains insufficient and this mycotoxin is not yet subject to any legislation even though this is the case for other similar mycotoxins (i.e. DON). In addition, a modified form of NIV, nivalenol-3-glucoside (NIV3G) can originate as a result of the wheat detoxification system by NIV conjugation with glucose. Preliminary investigations in animals showed that NIV3G toxicity is lower than that of NIV, but additional studies are needed to establish the impact of NIV3G in humans.

Besides NIV, F. poae is also able to biosynthesize another type B trichothecene (fusarenon-X), as well as type A trichothecenes [T-2 toxin, HT-2 toxin (even if with low levels and frequencies), diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), scirpentriol, neosolaniol] and non-trichothecene secondary metabolites such as enniatins and beauvericin (BEA). In addition to the mycotoxin risk, the presence of F. poae could also have an impact on baking quality by affecting and degrading the gliadin protein fraction.

The routes by which conidia of F. poae establish infections in cereal spikes are not clearly understood. Anthesis is considered the most susceptible growth stage for Fusarium infection of wheat. The opening of florets and the complete extrusion of anthers are the main events that establish infections by allowing fungal hyphae to enter individual florets. However, F. poae might colonize spikes early, prior to their emergence from the boot. In addition to the anthers, Fusarium species can also infect wheat at the adaxial surface of glumes, as well as lemma or palea, probably partly as a result of the action of hydrolytic enzymes.

Infection timing is important for FHB disease occurrence, kernel development, symptom appearance and morphology, as well as for mycotoxin accumulation in the grain. In the case of F. graminearum, the period of maximum receptivity of field-grown wheat to successful infections was reported to be up to 10 days after mid-anthesis, with the duration of susceptibility also related to the environmental conditions and host phenotype. Several field studies reported that high humidity at stages later than flowering can cause late F. graminearum infections with low levels of symptoms on grain but a high DON content.

Indeed, grain colonization by different Fusarium species was found to be differently affected by inoculation timing during the 10 days of maximum receptivity after early anthesis. In greenhouse conditions, F. poae and its mycotoxins were less influenced by infection timing than the more aggressive species F. graminearum and DON. In a 2-year field experiment conducted in Northern Italy, in which durum wheat spikes were inoculated with F. poae, NIV contamination was relatively similar in level across multiple infection timings, whereas fungal colonization was affected by infection timing, although variation in trends between years was reported.

Building on this previous work, the present study aimed to explore the influence of infection timings on F. poae colonization of bread wheat spikes and the resulting mycotoxin accumulation in grain. A 2-year field experiment was conducted in Central Italy, as well as an experiment in a controlled environment. In particular, the present study investigated the effects of inoculation with F. poae at growth stages from beginning of flowering to medium milk on: spike, kernel and lemma symptoms development/morphology; grain yield; fungal biomass distribution in the grain and chaff; secondary metabolites accumulation in the grain.
palea, lemma, rachilla, but not rachis nor awns) was hand-separated from the grain. For each inoculation timing, a stereomicroscope (SZX9; Olympus Corporation, Tokyo, Japan) was used to visually score grain and lemma (as representative of chaff material) for disease incidence and, for the grain only, to describe symptom expression. In particular, presence of these types of symptoms was visually scored on kernels: shrivelled, shrivelled + black, and black. Asymptomatic kernels were also enumerated. The incidence (%) of kernel symptom was calculated as the number of kernels with a specific type of symptom over the total number of kernels per replicate × 100. Moreover, grain weight for each inoculation timing was also determined, and the percentage of darkened lemmas was calculated.

Within each inoculation timing replicate, kernels and chaff material of the three spikes were bulked separately, finely ground by a laboratory mill (IMETEC, Azzano San Paolo, Italy) and split into two subsamples. The first one (both grain and chaff) was used to determine fungal biomass by a real-time quantitative polymerase chain reaction (qPCR), whereas the second one (only grain) was used to quantify F. poae secondary metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Field experiment

Plants of the commercial bread winter wheat cv. Ambrogio, which is susceptible to FHB, were grown during the wheat seasons 2017–2018 and 2018–2019 (hereafter called 2018 and 2019, respectively) in 1.5 × 6 m (9 m²) experimental plots, in a randomized block design experiment. Within each replicate block, the five inoculation timings and one non-inoculated treatment (a total of six treatments per block) were randomly assigned to plots. The experiment was arranged with three blocks for a total of 18 plots.

The experimental field was located at the FIELDLAB of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia (Papiano, Umbria, Italy, 42°57′N, 12°22′E, 165 m a.s.l.). Bread wheat was sown during the third week of November of both 2017 and 2018. A topdress fertilizer with urea (160 kg N ha⁻¹) was applied in addition to the pre-planting fertilizer application (75 kg P₂O₅ ha⁻¹). A post-emergence weed control was carried out at end-tillering stage (BBCH 29) with 3 L ha⁻¹ of Manta Gold (clopyralid 2.3% + fluoroxypr 6% + MCPA 26.7%; Syngenta, Basel, Switzerland) + 0.75 L ha⁻¹ of Axial Pronto (pinoxaden 5.05% + cloquintocet-mexyl 1.26%; Syngenta).

Wheat spikes were inoculated with F. poae at the same timings as for the climatic chamber experiment (BBCH 61, 65, 69, 71 and 75) each year, with a conidial suspension containing 1 × 10⁷ conidia mL⁻¹ and amended with 0.05% of Tween 20 (Sigma-Aldrich) using a hand-pump sprayer equipped with 500 L ha⁻¹ nozzles. In addition, to evaluate the possible presence of natural FHB infection, three non-inoculated plots were also included in the experiment.

FHB symptoms caused by F. poae were visually estimated 1 month after the first inoculation timing (BBCH 61) by determining, in each plot, both the average incidence (average % of spikes with symptoms per plot) and the average severity (average % spike area with symptoms per plot). FHB symptoms were then expressed as [(incidence %) × gravity %]/100).

Plots were harvested in their entirety at BBCH 92 to determine grain yield (adjusted to a kernel moisture content of 13% and expressed as t ha⁻¹), and grain was separated from chaff material using a spike threshers. Grain and chaff samples (500 g) from each plot were finely ground using a laboratory mill (IMETEC) and split into two subsamples. The first one (grain and chaff) was used to determine F. poae fungal biomass by qPCR, whereas the second one (the 2018 grain only) was used to identify and quantify F. poae secondary metabolites by LC-MS/MS analysis.

In addition, data on average temperature, minimum and maximum temperatures, rainfall and relative humidity were recorded daily by a weather station located at the FIELDLAB. For both years, weather data were acquired from 25 April to 10 June, the period during which inoculation occurred.

DNA extraction and quantification of F. poae DNA by qPCR

The qPCR assay used in the present study, both for climatic chamber and field experiments, has been described previously. To set up standard curves for the qPCR assays, preliminary DNA samples were extracted from pure F. poae colonies (FP 56sw10), healthy wheat kernels and chaff (cv. A416, for climatic chamber experiment and cv. Ambrogio for field experiment), as described previously. After DNA quantification with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), dilution series from 5 ng to 0.05 pg of F. poae DNA and from 50 ng to 5 pg of the bread wheat (grain and chaff) DNA, with a dilution factor of 10, were produced to set up standard curves that were processed in each qPCR assay. Standard curves were generated by plotting the logarithmic values of known DNA quantities versus the corresponding cycle threshold (Ct) values. The limit of detection (LOD) of F. poae DNA was 0.05 pg. Separate standard curves were developed for the field and climatic chamber experiments. Analyses were carried out using species-specific primers for the quantification of F. poae (FpoaeAs1 fwd 5'-ACCGAATCTCAACTCGCTTT-3'; FpoaeA98 rev 5'-GTCTGTCAGGCATATGGCAAGAT-3'; whereas translation elongation factor 1α (tef1α) primers (Hor1 fwd 5'-TCTCTGGGTGAGGGTGAC-3'; Hor2 rev 5'-GGCCCTGTACCAGT-CAGGTT-3') were used for the quantification of wheat DNA (grains and chaff).

The DNA from the experimental samples was extracted following the method described previously. For extractions, 4 g of grain and 2 g of chaff were used for both cv. A416 in the climatic chamber experiment and cv. Ambrogio in the field experiment. The extracted DNA was quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific) and the concentration of each DNA sample was adjusted to 30 ng μL⁻¹. The qPCR assays were carried out in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, California, USA), in accordance with the protocol described previously, using primers for the quantification of F. poae DNA and wheat DNA (grain and chaff). The fungal DNA in the wheat grain and chaff was expressed as the ratio of the F. poae DNA (pg) to the plant DNA (ng).

Detection and quantification of fungal secondary metabolites

One gram of each ground grain sample from both the climatic chamber experiment and the 2018 field experiment was extracted using 4 mL of extraction solvent (acetonytirile-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 Turbo Ion Spray electrospray ionization source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C18-column, 150 × 4.6 mm inner diameter, 5 μm particle size, equipped with a C18 4 × 3 mm inner diameter security guard cartridge (all from...
Phenomenex, Torrance, CA, USA). The chromatographic method, as well as chromatographic and mass spectrometric parameters, have been described previously.\textsuperscript{19,20} Quantification was performed via external calibration using serial dilutions of a multi-analyte stock solution. The results were corrected for apparent recoveries obtained for wheat.\textsuperscript{20} The accuracy of the method is verified on a continuous basis by regular participation in proficiency testing schemes. The LOD and limit of quantification for each \textit{F. poae} secondary metabolite detected by LC–MS/MS are reported in the Supporting information (Table S1).

### Statistical analysis

The experiment in the climatic chamber had three replicates. For each of the following analyses, one-way analysis of variance was conducted, and inoculation timing was treated as the independent variable. For FHB symptom severity, percentage data on symptom types in symptomatic grain, as well as the percentage of asymptomatic kernels, were evaluated as the dependent variable. FHB symptoms caused by \textit{F. poae} and scored at 7 days post each inoculation timing are presented as averages. Hand-harvested grain was also analysed as a dependent variable. As for the chaff, the percentage of symptomatic lemmas was evaluated as a dependent variable. \textit{F. poae} DNA accumulation in grain and chaff was analysed using pg of fungal DNA per ng of wheat DNA as the dependent variable. For secondary metabolite accumulation in grain, quantity of secondary metabolites (μg kg\textsuperscript{-1}) was the dependent variable.

For the field experiment, data on FHB symptoms and grain yield were subjected to one-way analysis of variance by considering inoculation timing the independent variable and symptoms (%) or grain yield in t ha\textsuperscript{-1} as dependent variables. The same type of analysis described for the climatic chamber experiment was carried out for \textit{F. poae} DNA accumulation in grain and chaff and for fungal secondary metabolite accumulation in the grain.

To test pairwise contrasts, in each case, Tukey's honestly significant difference (HSD) multiple comparison tests were performed (\(P \leq 0.05\)) using \textit{DAASTAT}, version 1.0192.\textsuperscript{19} Finally, the correlations between \textit{F. poae} secondary metabolites and fungal biomass were evaluated using the Pearson correlation coefficient (\(r\)), followed by Student's \(t\)-test.

### RESULTS

#### Climatic chamber experiment

\textit{Fusarium poae} inoculation timing considerably affected FHB symptom severity on spikes (Fig. 1a). The growth stages of beginning and full flowering (BBCH 61 and 65) favoured symptom development. These early inoculations resulted in similar severities (\(P \geq 0.05\)) of 60.3\% and 71.8\%, respectively. Inoculations at full flowering caused significantly higher symptom severity than did those at the three later infection timings (BBCH 69 = 47\%, BBCH 71 = 38.6\% and BBCH 75 = 35.1\%; \(P \leq 0.05\)). Similarly, symptom severity following inoculation at BBCH 61 was significantly higher than that after inoculation at BBCH 71 and BBCH 75 (\(P \leq 0.05\)), although not after inoculation at BBCH 69 (\(P \geq 0.05\)). The three latest inoculation timings resulted in similar FHB symptom severities (\(P \geq 0.05\)). Thus, the observed gradient for FHB symptom severity was: BBCH 65 ≥ BBCH 61 ≥ BBCH 69 ≥ BBCH 71 = BBCH 75. No FHB symptoms were detected on control spikes.

Under controlled environmental conditions, infection timing had an impact on wheat yield (Fig. 1b). Grain weight in early-inoculated spikes (BBCH 61 and BBCH 65) was significantly lower than that in spikes inoculated at the two latest timings (BBCH 71 and BBCH 75) or in non-inoculated spikes (\(P \leq 0.05\)). Yield in the BBCH 69 treatment did not differ from that following earlier or later inoculations, nor were yields following infections at BBCH 69 to 75 significantly different from that of the non-inoculated control (\(P \geq 0.05\)).

Grain harvested at senescence from wheat spikes inoculated at different growth stages with \textit{F. poae} included kernels that were shrivelled, black, or a combination of shrivelled and black, as well as asymptomatic kernels (Fig. 2). Interestingly, \textit{F. poae} inoculation timing considerably affected the incidence of symptoms on kernels. For example, the percentage of shrivelled kernels significantly decreased (\(P \leq 0.05\)) from early inoculation timings (BBCH 61 = 37.5\% and BBCH 65 = 37.1\%) to later inoculation timings (BBCH 69 = 25.9\%, BBCH 71 = 21.1\% and BBCH 75 = 16.6\%). Similarly, the percentage of kernels that simultaneously showed shrivelling and blackening symptoms decreased significantly (\(P \leq 0.05\)) from BBCH 61–65 (33.3\% and 33.7\%) to BBCH 69–71–75 (11.4, 5.7 and 0\%). However, a significant increase of kernels with only blackening symptoms was observed among spikes inoculated at the later timings (BBCH 69 = 28.2\% and BBCH 71 = 28.2\%) compared to the earlier timings (BBCH 61 = 8.6\% and BBCH 65 = 13.4\%). Blackening, whether with or without shrivelling, was absent on grain obtained from spikes inoculated at BBCH 75. None of the disease symptoms was observed in kernels obtained from non-inoculated spikes. The percentage of asymptomatic kernels significantly increased from early to late \textit{F. poae} infection timing, with the two earliest timings resulting in significantly lower percentages than the three latest timings (\(P \leq 0.05\)).

The incidence of shrivelled and shrivelled + black kernels following early inoculations (BBCH 61 and BBCH 65) was significantly higher (\(P \leq 0.05\)) than the incidences of only black or asymptomatic grain. Conversely, starting with the late-flowering inoculation (BBCH 69), the incidence of asymptomatic grain was significantly higher (\(P \leq 0.05\)) than the incidence of any disease symptom type.

Lemmas that had been hand-separated from grain and observed with a stereomicroscope showed varying darkening intensity depending on \textit{F. poae} infection timing (Fig. 3). Lemma darkening was absent in spikes inoculated at the latest two timings, BBCH 71 and 75, whereas it was detected on spikes inoculated at the three earlier timings. The dark area was particularly developed on the internal side of the lemma, which is in close contact with the kernel (Fig. 3). The percentage of darkened lemmas was highest following inoculation at early flowering (43.3\%), dropped significantly for each of the next two timings (BBCH 65 = 22.7\%, BBCH 69 = 15.3\%; \(P < 0.05\)) and fell to zero by the fourth timing, BBCH71 (see Supporting information, Fig. S1).

\textit{Fusarium poae} DNA in grain and chaff

The \(R^2\) value calculated from the linear equation of the \textit{F. poae} standard curve was 0.99. Reaction efficiency obtained from the \textit{F. poae} linear equation was 100\%. The dissociation curve analysis showed specific amplification products in the presence of pure fungal DNA (standard curves) and in the presence of \textit{F. poae} DNA (samples). No target amplification was detected in the negative controls. Therefore, the Ct values used to quantify fungal biomass were those for which dissociation curve analysis showed the presence of specific amplification products. \textit{F. poae} analyzed by qPCR was detectable through the presence of the DNA
accumulation (pg of fungal DNA ng of plant DNA⁻¹) both in grain and in chaff manually separated immediately after the harvest at BBCH 92.

In the climatic chamber experiment, *F. poae* DNA accumulation in both grain and chaff was strongly affected by infection timing (Fig. 4), showing a decrease from early to late application of inoculum. The pattern of *F. poae* DNA accumulation in both grain and chaff was: BBCH 61 = beginning of flowering; BBCH 65 = full flowering; BBCH 69 = end of flowering; BBCH 71 = watery ripe; BBCH 75 = medium milk. No significant difference between *F. poae* accumulation after BBCH 71 and BBCH 75 infection timing was observed ($P \geq 0.05$). No *F. poae* DNA was detected in grain from control spikes. Similarly, in chaff, biomass declined as inoculation timing became later, and *F. poae* was not detected in the chaff controls spikes. Levels of *F. poae* DNA accumulated in chaff were significantly higher than those detected in grain for all inoculation timings (BBCH 61: $P = 0.003$; BBCH 65: $P = 0.006$; BBCH 69: $P = 0.05$; BBCH 71: $P = 0.009$; BBCH 75: $P = 0.025$).
The fungal secondary metabolites detected in grain from spikes of bread wheat inoculated with *Fusarium poae* in the climatic chamber at various timings are summarized in Table 1. Overall, the highest accumulation of *F. poae* secondary metabolites (NIV, MAS, DAS and BEA) was detected after the first two inoculation timings (BBCH 61 and 65) with a decrease in response to the later infections (BBCH 69, 71, and 75). In detail, NIV accumulation followed the gradient: BBCH 61 ≥ 65 > 69 ≥ 71 ≥ 75. NIV levels were significantly higher after the first two infection timings (P ≤ 0.05) than...
after the other timings. Because of the ability of wheat to detoxify NIV, NIV3G was also detected in the grain. This metabolite followed the same trend as NIV. No NIV3G was detected at BBCH 75 timing. The MAS and DAS accumulation gradients were similar (BBCH 61 ≥ 65 ≥ 69 ≥ 71 ≥ 75), with the level detected at BBCH 61 higher than that recorded from BBCH 69 onward. No DAS was detected for the BBCH 75 timing. Finally, also BEA accumulation showed a comparable pattern of other mycotoxins: BBCH 61 ≥ 65 ≥ 69 ≥ 71 ≥ 75. None of the fungal secondary metabolites previously described was detected (< LOD) in grain collected from non-inoculated wheat spikes (control).

Field experiment
FHB symptoms and grain yield
In the 2-year field experiment, FHB symptoms were estimated at BBCH 79, 30 days post the first inoculation timing (BBCH 61) and before senescence prevented them from being visually distinguished. During the 2018 experiment, a descending trend in symptom severity was observed from plots inoculated at BBCH 61 to plots inoculated at BBCH 75, with the gradient: BBCH 61 ≥ 65 ≥ 69 ≥ 71 = 75 ≥ control (Fig. 5). Symptoms observed after the last three infection timings were similar to those observed in control plots under natural infection pressure.

By contrast, in the 2019 field experiment, greater FHB symptom severity was observed in plots inoculated at BBCH 65 than in the remaining timing treatments, which were all statistically indistinguishable (P ≤ 0.05) (Fig. 5). In 2019, the non-inoculated control plots had three times the disease severity as those of 2018 (10% vs. 2.7%), which may have reflected a higher background level of spike blight infections from naturally occurring fusaria.

In summary, in both years of the field experiment, only early inoculation timings (BBCH 61 alone in the first year and BBCH 65 in both years) resulted in FHB severities significantly higher than the non-inoculated controls. These results suggested that...
at these two early growth stages, F. poae infections can contribute significantly to FHB symptoms under field conditions. It is possible that various naturally occurring Fusarium spp. contributed to the FHB epidemics in addition to F. poae, particularly in 2019.

Despite the differences in FHB symptom severity, the inoculation with F. poae at different growth stages did not significantly affect grain yield (see Supporting information, Fig. S2) in either year ($P > 0.05$; data not otherwise shown). Yields for all timing treatments were statistically indistinguishable from each other and from the non-inoculated control. Average grain yield was 5.31 t ha$^{-1}$ in 2018 (from 4.79 to 5.66 t ha$^{-1}$) and 5.39 t ha$^{-1}$ in 2019 (from 5.05 to 5.56 t ha$^{-1}$).

Fusarium poae DNA in grain and chaff

In general, F. poae DNA in grain resulting from the different spike inoculation timings in the 2-year field experiment (Fig. 6) reflected the pattern of visual symptoms (Fig. 5). In 2018, F. poae in grain after the earliest infection timing (BBCH 61) was significantly higher than the levels resulting from all the other infection timings and in the non-inoculated control ($P \leq 0.05$) (Fig. 6a). In 2019, a significant increase ($P \leq 0.05$) of F. poae biomass was detected in grain collected from spikes inoculated at BBCH 65 compared to that recovered following other inoculation timings and the control (Fig. 6b). Indeed, fungal DNA levels among the later BBCH 69, 71, and 75 treatments were never significantly different from control levels ($P \geq 0.05$).

In summary, in the 2-year field experiment, only early inoculation timings (BBCH 61 the first year and BBCH 65 the second year) resulted in fungal DNA significantly higher than in the non-inoculated control. This indicated that only F. poae infections during the beginning and middle stages of flowering significantly affected F. poae colonization of grain under field conditions.

Fusarium poae DNA was also analyzed by qPCR in chaff material. Just as in grain, F. poae DNA levels in chaff followed the pattern apparent in the visual symptoms (Fig. 6). In 2018 chaff samples, F. poae DNA from BBCH 61 infections was significantly greater than that from other timings as well as from the non-inoculated control, which were all statistically indistinguishable from each other ($P \leq 0.05$) (Fig. 6a). In 2019, although F. poae DNA in chaff followed a similar pattern as for grain in the same year, the numerically higher level in the BBCH 65 treatment was not statistically different from the levels of the BBCH 61, BBCH 69 and BBCH 71 timings (Fig. 6b). BBCH 65 was the only inoculation timing that produced a significantly higher chaff biomass level than the control ($P \leq 0.05$).

As in the climatic chamber experiment, the levels of F. poae DNA in chaff material were always numerically higher than those detected in grain for each inoculation timing in both experimental years. However, significant differences were observed only at BBCH 61 ($P = 0.026$) in 2018 and at BBCH 65 ($P = 0.026$), BBCH 71 ($P = 8 \times 10^{-6}$) and BBCH 75 ($P = 0.002$) in 2019.

Fusarium poae secondary metabolites in grain

As in the climatic chamber experiment, the highest levels of F. poae secondary metabolites from the field experiment were detected in grain from spikes inoculated at the earliest infection timings (BBCH 61 and 65) (Table 2). In detail, NIV was detected in grain only after the BBCH 61 and BBCH 65 inoculation timings, with a significant difference ($P \leq 0.05$) between them. From BBCH 69 to BBCH 75, as well as in the control, NIV was not detected (< LOD). By contrast to the controlled-environment experiment, no NIV3G was detected in grain from the field experiment.

MAS and BEA followed the NIV trend, with detection only at BBCH 61 and BBCH 65 and the highest amount ($P \geq 0.05$) in grain from the first time point. These two mycotoxins were under the LOD for all the other timings, with the exception of BBCH 75, where they were detected in very small amounts. By contrast with the climatic chamber experiment, no DAS was detected in grain from the field experiment. In summary, in the 2018 field experiment, only infections from early inoculation timings (BBCH 61) resulted in mycotoxin levels significantly higher than from the non-inoculated control. This suggested that at that growth stage, F. poae infection can significantly affect the accumulation of F. poae secondary metabolites (NIV, MAS, BEA) in wheat grain under field conditions.

Weather conditions in 2018 and 2019

Data on temperature, relative humidity and rainfall were collected from 25 April to 10 June of both 2018 and 2019 at the
Figure 6. Fungal DNA accumulation (pg of fungal DNA ng grain DNA $^{-1}$), detected by qRT-PCR, in bread wheat grain and chaff harvested at BBCH 92 from spikes inoculated with Fusarium poae at one of five growth stages or from non-inoculated plots in a field experiment conducted in 2018 (a) and in 2019 (b). Values are means of three biological replicates. Within an inoculation timing (a, b) or between inoculation timings (A–C), means with the same letters are not significantly different at $P \leq 0.05$ based on Tukey’s HSD test. BBCH 61 = beginning of flowering; BBCH65 = full flowering; BBCH69 = end of flowering; BBCH71 = watery ripe; BBCH75 = medium milk.
The experimental station where the field experiment was carried out (Fig. 7). Rainfall was similar in average daily amount and total amount in 2018 (2.7 and 114.2 mm, respectively) and 2019 (2.8 and 132 mm, respectively). The 2 years were similar also in relative humidity (average levels of 72.2% in 2018 and 71.1% in 2019). However, differences in temperature were observed, with an average daily temperature of 19.3°C in 2018 and 16°C in 2019. This is likely the reason that wheat growth stages used for *F. poae* inoculations occurred with a lag of 6–10 days in 2019 compared to in 2018 (Fig. 7).

Considering weather conditions at the inoculation dates (especially the interval 0–48 h post-inoculation) (Fig. 7), it can be seen that, in 2018, around BBCH 61, there was a longer interval of continuous wetting, stretching from 48 h pre-inoculation to 72 h post-inoculation.

#### Table 2. Concentrations of secondary metabolites in bread wheat grain collected at BBCH 92 from spikes inoculated with *Fusarium poae* at different growth stages or from non-inoculated (control) spikes in the 2018 field experiment

| Inoculation timings* | NIV\(^v\) | SE | MAS\(^x\) | SE | BEA\(^y\) | SE |
|----------------------|-----------|----|-----------|----|-----------|----|
| BBCH 61              | 489 a     | 104| 37.2 a    | 6.49| 18.7 a    | 2.79|
| BBCH 65              | 73.6 b    | 29.9| 5.10 b    | 5.10| 1.81 b    | 1.02|
| BBCH 69              | < LOD\(^z\) b | < LOD b | < LOD b | < LOD b |
| BBCH 71              | < LOD b   | < LOD b | < LOD b | < LOD b |
| BBCH 75              | < LOD b   | 2.6 b | 2.62      | 0.49 b | 0.49 |
| CONTROL              | < LOD b   | < LOD b | 1.77 b    | 1.09 |

*BBCH 61, beginning of flowering; BBCH65, full flowering; BBCH69, end of flowering; BBCH71, watery ripe; BBCH75, medium milk.

\(^v\) Metabolite concentrations were detected by liquid chromatography tandem mass spectrometry analysis and are the mean ± SE of three biological replicates. Within a column, values with the same lowercase letter are not significantly different at \(P \leq 0.05\) based on Tukey’s HSD multiple comparison test.

\(^x\) NIV, nivalenol.

\(^y\) MAS, monoacetoxyscirpenol.

\(^z\) BEA, beauvericin.

\(^z\) LOD, limit of detection.
post-inoculation. At BBCH 65, no rainfall occurred on the day of inoculation, although two substantial rain events took place in the subsequent 48 h. The BBCH 69 inoculation was the only one for which no rainfall events occurred in the 48 h post-inoculation period. A rainfall event occurred during the BBCH 71 inoculation day, followed by another the day after. Finally, at BBCH 75, moderate rainfall events occurred respectively at 0, 24 and 48 h post-inoculation.

During 2019, negligible rainfall events occurred at the BBCH 61 timing. At BBCH 65, again only a light rain occurred the day of inoculation; however, two substantial rain showers occurred at 72 and 96 h post-inoculation. At BBCH 69, a light rain occurred the day of inoculation. At BBCH 71, after two modest rain events at 0 and 24 h post-inoculation, a major event occurred. These conditions increased relative humidity, and then three further moderate/light rain events in the 72- to 120-h post-inoculation period additionally increasing the humidity. Lastly, at BBCH 75, no rainfall occurred in the interval 0–48 h post-inoculation.

Correlation between quantified *F. poae* DNA and secondary metabolites in the grain

In the climatic chamber experiment, *F. poae* DNA accumulation showed a positive correlation with secondary metabolites detected in grain, across growth stages. In particular, a significant positive relationship was observed between *F. poae* DNA and NIV (*r* = 0.96; *P* = 8.7 × 10⁻⁵), NIV3G (*r* = 0.94; *P* = 6.5 × 10⁻⁴), MAS (*r* = 0.95; *P* = 1.1 × 10⁻⁴) and DAS (*r* = 0.92; *P* = 2.3 × 10⁻³) (results shown for NIV only) (Fig. 8a). Also, BEA, even if less strongly, was positively related to fungal DNA (*r* = 0.65; *P* = 6.6 × 10⁻²).

In the 2018 field experiment, a strong positive association between NIV accumulation and *F. poae* DNA in grain was also observed (*r* = 0.93; *P* = 5.2 × 10⁻²) (Fig. 8b). Finally, a positive, but not significant, correlation between *F. poae* DNA and MAS (*r* = 0.92; *P* = 1.7 × 10⁻¹) and BEA (*r* = 0.85; *P* = 2.6 × 10⁻¹) was also observed.

**Figure 8.** Correlation between nivalenol (µg kg⁻¹) and *Fusarium poae* colonization (pg of fungal DNA ng wheat DNA⁻¹) following inoculation at five growth stages in a climatic chamber experiment (a) and a 2018 field experiment (b). The three replicates are shown separately. BBCH 61 = beginning of flowering; BBCH65 = full flowering; BBCH69 = end of flowering; BBCH71 = watery ripe; BBCH75 = medium milk.
DISCUSSION

Fusarium poae is an FHB pathogen that needs further attention because of the expansion of its presence in many wheat cultivation areas and its ability to produce mycotoxins, especially NIV, that can negatively affect human and animal health. The increasing occurrence of F. poae could be associated with various factors such as climate change and/or selection pressure caused by common fungicides worldwide. Furthermore, the timing of infection may play a role in FHB development, favouring some Fusarium species to the detriment of others. For these reasons, the present study focused on the effect of different infection timings on F. poae colonization and its secondary metabolites in bread wheat grain both in a controlled-environment study and in field conditions.

The classical model for Fusarium spp. colonization of wheat spikes suggests that the infection usually starts from anthers. Therefore, the capability of F. poae to infect wheat was investigated during five wheat growth stages. The first three differed in the number and age of extruded anthers (BBCH 61, BBCH 65 and BBCH 69), whereas the last two were post-anthesis (BBCH 71 and BBCH 75). The use of a single strain of F. poae does not allow a general conclusion to be drawn at the species level; however, our results can provide insight into the timing of F. poae infection most likely to cause damage.

The results obtained from the climatic chamber experiment suggest that, under those experimental conditions, infection timing influences F. poae colonization as well as its secondary metabolite accumulation in bread wheat grain. Under controlled conditions, F. poae had a particular advantage at early infection timings (beginning of flowering and full flowering), whereas infection at watery-ripe and medium milky stages strongly reduced F. poae colonization. Finally, the end of anthesis appears to be an intermediate stage in terms of F. poae colonization efficiency. This suggests that F. poae, at least under controlled conditions, performs best in the presence of anthers at the earlier stages of their development. This may be because the anthers' composition and level of extrusion make them more receptive to colonization by the fungus.

The pattern of infection previously described was generally confirmed by all parameters evaluated in the climatic chamber experiment, such as FHB symptoms on spikes, the incidence of shrivelling, presence of shrivelling) were mainly detected after spike infection. Interestingly, the climatic chamber experiment also showed that, although typical FHB symptoms of bleaching and shrivelling of kernels were mainly detected in connection with early infection timing, black kernels (without bleaching and shrivelling of kernels were mainly detected in 2018 at BBCH 61 and BBCH 69). In both types of experiments in the present study, it was apparent that, at each infection timing, F. poae accumulated a larger share of its biomass in chaff than in grain. This difference remained consistently significant in the second year of experimentation for all infection timings, except at BBCH 61. In addition, this was observed also in 2018 at BBCH 61 and BBCH 69, whereas, at other BBCHs, no differences between the two tissues were statistically significant, probably as a result of the higher variation in the field experiments, which is common compared to controlled environments. In both types of experiments in the present study, it was apparent that, at each infection timing, F. poae accumulated a larger share of its biomass in chaff than in grain. This confirms the previous observations in which F. poae was commonly isolated from cereal glumes.

Frequently, a strong correlation between mycotoxins and the DNA content of single Fusarium species has been reported. The positive association between F. poae fungal biomass and its secondary metabolites in grains was detected also in this controlled-environment experiment. The highest levels of NIV in particular were detected following infections during flowering, although infections at watery-ripe also led to detectable levels of NIV and other mycotoxins. Interestingly, the climatic chamber experiment also revealed the presence of NIV3G in grain after all inoculation timings except for BBCH 75, indicating that wheat detoxification of NIV occurs across various infection timings. It is known that, usually, NIV3G concentrations in grain are related to starting levels of NIV; in other words, the higher level of NIV, the more NIV3G is detected in grain. In the present study, we detected the same trend but calculating the [NIV:NIV3G] ratio, we also observed an increasing ratio from early to late F. poae infection timings (7:1 at BBCH 61 and BBCH 65; 10:1 at BBCH 69; 15:1 at BBCH 71). We hypothesize that NIV transformation to
NIV3G may be more efficient when NIV concentrations are higher, or possibly that conversion of NIV by the wheat plant is not triggered as actively when infections occur later in spike development.

By contrast to the observations in the present study, previous studies in a controlled environment (greenhouse) did not show evidence that bread wheat infection timing affected extent of *F. poae* colonization or mycotoxin accumulation. However, in addition to the difference between the greenhouse and the climatic chamber, there were substantial differences among the studies with respect to factors such as infection timings, inoculum doses, *F. poae* strains and bread wheat genotypes. These differences may have played a role in the observed effects of spike infection timings on *F. poae* and its mycotoxin accumulation. For example, the effect of infection timing differed even among strains of *F. graminearum* and of *F. culmorum*, as well as among different barley genotypes.

Our 2-year field experiment confirmed that, under our conditions, infection timing influenced *F. poae* colonization as well as the accumulation of its secondary metabolites in bread wheat grain. Here again, early infection timings (BBCH 61 in 2018 and BBCH 65 in 2019) were the most favourable for *F. poae* colonization and mycotoxin accumulation. However, the time window of bread wheat susceptibility in the field was short compared to that under controlled conditions. Indeed, in both years, only one infection timing (beginning of flowering in 2018 and full flowering in 2019) differed significantly from all the others. This restricted time-window of host susceptibility detected in the field, compared to that under controlled conditions, could be related to the many variables in an outdoor environment that may reduce favourability for a pathogen’s infection and development.

A positive correlation between *F. poae* biomass and NIV accumulation in grain was also observed in the field experiment. Unlike in the climatic chamber experiment, a detectable level of NIV under 2018 field conditions was recorded only after the early *F. poae* infection timings (BBCH 61 and 65), whereas later infections (BBCH 69, 71 and 75) did not lead to NIV accumulation in grain. Thus, the time window for NIV accumulation in field-grown bread wheat was shorter than that observed under controlled conditions. However, *F. poae* fungal biomass and NIV were detected in grain harvested from durum wheat spike inoculated at later stages, suggesting that the window of susceptibility to *F. poae* and NIV accumulation could be more extended. Again, differences in *F. poae* strains, host genotypes and weather conditions may explain the varying results of these experiments. For those reasons, it would be useful to investigate the impact of different infection timings on *F. poae* colonization and mycotoxin accumulation in bread wheat grain using different fungal strains and host genotypes, as well as multiple field locations, to obtain a more comprehensive view.

Increased performance of *F. poae* at early flowering in 2018 and full flowering in 2019 was generally confirmed by the majority of parameters evaluated in field experiments such as FHB symptoms on the spike, fungal biomass in grain and chaff, and secondary metabolite accumulation (analyzed only in 2018). However, *F. poae* infection did not reduce grain yield in either year of the field experiment, even after early infections that produced more greater symptoms and colonization. This confirms the relatively low impact of this ‘weak’ FHB pathogen on wheat yield in the field. The absence of significant wheat yield reduction by *F. poae* infection in greenhouse and field conditions has been reported previously.

The present study showed that, in a Central Italy field environment and in bread wheat, early or full flowering (BBCH 61 or 65) can be considered as the infection timings when *F. poae* exhibits maximum efficiency of colonization and mycotoxin accumulation relative to infection at other growth stages. For comparison, in the recent Northern Italy field experiment that utilized *F. poae* artificial inoculation of durum wheat spikes, fungal colonization was affected by infection timing and was particularly favoured by inoculation at full flowering, although the results were variable between the 2 years. In that study, NIV contamination was more consistent across different infection timings compared to *F. poae* presence.

In the present study, because the experimental treatments and agricultural practices carried out during the two growing seasons were the same, the variation between the 2 years in symptom severity and fungal biomass in grain and chaff is likely related to different weather conditions. For example, at BBCH 61, weather conditions were more conducive to *Fusarium* infection in 2018 than in 2019. There was rain in the first year during and directly after the inoculation, and humidity was relatively high (77.8–81.7%). In the second year, there was less precipitation and a lower relative humidity at that inoculation timing, as well as a lower average temperature. This could help account for the relatively low levels of symptoms and fungal biomass from the first inoculation in 2019 compared to 2018. However, a study repeated for more than 2 years could provide more information about the interaction between infection timing and weather with respect to determining the susceptibility level of bread wheat to *F. poae*. Nevertheless, both BBCH 61 and BBCH 65 showed also the highest susceptibility to *F. poae* under controlled conditions. Therefore, considering all the evidence, it is probable that the stages from early flowering up to full flowering comprise the most susceptible growth stages of bread wheat to *F. poae*.

**CONCLUSIONS**

This research has extended our knowledge of *F. poae*, which is one of the most common FHB casual agents worldwide. Based on controlled conditions and two years of field trials conducted in Central Italy, the results indicate that infection timing appears to influence *F. poae* colonization and secondary metabolite accumulation in bread wheat. In particular, the study shows that, within the period from BBCH 61 to 75, early infections (beginning of flowering and full flowering) led to the highest levels of *F. poae* fungal biomass and its mycotoxins (especially NIV) in grain compared to infections at the late flowering, watery-ripe and medium milk stages. These results add to our understanding of *F. poae* epidemiology, the dynamics of NIV and other *F. poae* secondary metabolites in grain, and strategies for *F. poae* management, such as the correct timing of fungicide applications.

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**SUPPORTING INFORMATION**

Supporting information may be found in the online version of this article.

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