Effect of Infection Timing on Fusarium Head Blight and Mycotoxin Accumulation in Open- and Closed-Flowering Barley

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ABSTRACT

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Barley has two flowering types, chasmogamous (open-flowering) and cleistogamous (closed-flowering). We examined the effect of the timing of *Fusarium graminearum* infection on Fusarium head blight (FHB) and mycotoxin accumulation in barley cultivars with different flowering types using greenhouse experiments. In the first experiment, 13 cultivars were spray inoculated at two different developmental stages, and the severity of FHB was evaluated. The effect of the timing of infection differed among cultivars. Cleistogamous cultivars were resistant at anthesis but susceptible at 10 days after anthesis, whereas chasmogamous cultivars were already susceptible at anthesis. In the second experiment, five cultivars

were inoculated at three different developmental stages and the concentrations of deoxynivalenol (DON) and nivalenol (NIV) in mature grain were analyzed. Cleistogamous cultivars accumulated more mycotoxins (DON and NIV) when inoculated 10 or 20 days after anthesis than when inoculated at anthesis, whereas chasmogamous cultivars accumulated more mycotoxins when inoculated at anthesis. Thus, the most critical time for *F. graminearum* infection and mycotoxin accumulation in barley differs with cultivar, and likely is associated with the flowering type. Late infection, even without accompanied FHB symptoms, was also significant in terms of the risk of mycotoxin contamination.

Additional keywords: anther, cleistogamy, *Gibberella zeae*, *Hordeum vulgare*, scab, tricothecene.

Fusarium head blight (FHB) or scab, which is caused by several *Fusarium* spp., primarily *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), is a widespread and destructive disease of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (18,22,32). This disease infects spikes and reduces grain yield and quality. Moreover, *Fusarium* spp. that cause FHB produce mycotoxins such as deoxynivalenol (DON), nivalenol (NIV), and zearalenone, which are toxic to humans and animals. Contamination of barley and wheat by these mycotoxins has been reported worldwide (25,37,38).

F. graminearum can be divided into two chemotaxonomic groups: the DON chemotype and NIV chemotype (9,13,30,34). DON is the most prevalent mycotoxin in cereals (4,25) and DON chemotypes of *Fusarium* spp. prevail worldwide, whereas NIV chemotypes are more restricted (12,21); therefore, greater attention generally is focused on DON than NIV as a tricothecene mycotoxin. A provisional maximum tolerable daily intake (PMTDI) of 1 µg of DON per kilogram of body weight was established by the Joint FAO/World Health Organization Expert Committee on Food Additives in 2001 (4). However, NIV is also an important *Fusarium* mycotoxin that frequently occurs worldwide (25,37) and commonly co-occurs with DON (25,37,38, 43,44). Moreover, NIV is more toxic to animals than DON (28,40). Accordingly, control measures that are effective for both NIV and DON should be established.

To control FHB and mycotoxin contamination effectively, the critical timing of infection must be known. Wheat is most sus-

ceptible to FHB during anthesis (1,22,26,35), which likely is due to anther extrusion at that stage, because the initial *Fusarium* infection commonly occurs on extruded anthers (26). Whereas wheat is generally chasmogamous (open-flowering type), barley has both chasmogamous and cleistogamous (closed-flowering type) cultivars; in the latter, the florets do not open and anthers are not extruded at anthesis.

In Japanese barley cultivars, there is a strong association between row type and flowering type; most two-rowed cultivars are cleistogamous, whereas most six-rowed cultivars are chasmogamous. In most cases, the former are relatively resistant and the latter are susceptible to FHB (42), which is consistent with the general observation that two-rowed types are more resistant than six-rowed types (3,7,17,32,36,47). Cleistogamy, rather than the row type, contributes to the resistance of barley cultivars to FHB, at least for infection that occurs at anthesis (42).

In our recent preliminary tests, however, high levels of DON and NIV contamination occurred, even in resistant two-rowed cleistogamous cultivars. In cleistogamous cultivars, anthers do not protrude at anthesis; however, they may be pushed out from the tips of florets by the developing grain several days after anthesis (2). In Japan, this phenomenon is observed commonly in cleistogamous cultivars. Thus, the timing of anther extrusion differs with flowering type. Therefore, the stage most susceptible to *Fusarium* infection may differ among barley cultivars.

McCallum and Tekauz (16) previously reported differential susceptibility to FHB at different developmental stages in four barley cultivars using spray inoculations. However, the effects of cultivar, timing of inoculation, and their interaction were significant for the colonization of seed by *F. graminearum* but not for visual symptoms of infection (16). The effect of the timing of *F. graminearum* infection on FHB in barley has not been well studied otherwise; to our knowledge, the effect of the timing of infection on mycotoxin contamination in barley has not been investigated.

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The objective of this study was to investigate the effect of the timing of infection by *F. graminearum* on FHB severity and the accumulation of DON and NIV in barley cultivars with different flowering types. Such knowledge will be helpful in developing effective control measures to reduce the risk of FHB and mycotoxin contamination in barley.

MATERIALS AND METHODS

Evaluation of FHB resistance at two developmental stages in barley cultivars (experiment 1). Thirteen barley cultivars differing in row type and flowering type (Fig. 1) were evaluated for FHB resistance at two developmental stages: anthesis and 10 days after anthesis (DAA). The experiment was conducted in Chikugo (33.1°N, 130.3°E), Fukuoka, Japan, in 2004 and 2005, although only 8 of the 13 cultivars were tested in 2004. The six-rowed cultivars were chasmogamous and the two-rowed cultivars were cleistogamous, except for the two-rowed chasmogamous ‘Satsuki Nijo,’ ‘Chevron,’ the only non-Japanese cultivar tested, is relatively resistant to FHB among six-rowed barley cultivars (3,17,27,32). The two-rowed cvs. Asahi 5, Seijo 17, and Nitta Nijo16 also are highly resistant (36). The other eight cultivars were representative samples of the current barley cultivars available for production in Japan. ‘Sanuki hadaka’ is a hull-less type, whereas the other 12 cultivars are all of the hulled type.

The cultivars were seeded in 15-cm-diameter plastic pots (four plants per pot) at the end of November 2003 and 2004, and were grown in a greenhouse under natural light until inoculation (March to April 2004 and 2005). The potting soil was prepared by mixing 25 liters of volcanic ash soil, 15 liters of sand, 10 liters of vermiculite, 10 liters of compost, and 75 ml of calcium magnesium carbonate. Each pot contained ≈1 liter of the mixed soil and was fertilized at the seedling growth stage (Zadoks growth stage [ZGS] 13) (46) and the stem elongation stage (ZGS 30 to 31) with 2.5 and 1.5 g, respectively, of N-P₂O₅-K₂O at 14-14-14.

F. graminearum isolate H-3 was used as the inoculum. Macroconidia of the isolate were produced by growing the fungus in mung bean liquid medium prepared as follows: 20 g of mung bean were boiled in 1 liters of water for 20 min, and 1 g of yeast extract was added; the broth then was strained through a colander and autoclaved. The macroconidia suspension obtained was filtered to remove hyphae and was centrifuged at 7,000 rpm for 10 min. After removal of the liquid medium, the pellet was suspended in distilled water and the macroconidia concentration was provisionally adjusted to 5×10^7 spores/ml and then stored at -80°C until use. On the days of inoculation, the macroconidia suspension was thawed at $22 \pm 2^\circ\text{C}$ (room temperature) and diluted to 5×10^5 spores/ml with distilled water.

Inoculation and FHB evaluation were performed using the ‘‘pot-plant’’ method (41,42), with some modification. Four (in

Fig. 1. Fusarium head blight (FHB) severity (0-to-100 scale) for 13 cultivars inoculated with *Fusarium graminearum* at two different developmental stages: anthesis and 10 days after anthesis (DAA) in 2004 and 2005. The severity of FHB was assessed 2 weeks after inoculation. Each point represents the mean of four (in 2004) or three (in 2005) replicates; bars represent standard errors; *, **, and *** indicate $P < 0.05$, 0.01 , and 0.001 , respectively, for differences between the two inoculation times (t test); ns = the difference was not significant; NT = not tested; cl = cleistogamous cultivar; and ch = chasmogamous cultivar.

Fig. 2. Fusarium head blight (FHB) severity caused by *Fusarium graminearum* at 20 days after anthesis (DAA) (0-to-100 scale) and mycotoxin concentration in mature grain for five barley cultivars and different inoculation times in 2004 and 2005. Numbers below the graph indicate the timing of inoculation in DAA. Each point represents the mean of three replicates; bars represent standard errors. Within cultivars, mycotoxin concentrations (the total of deoxynivalenol [DON] plus nivalenol [NIV]) with the same letter were not significantly different at $P = 0.05$ (Tukey-Kramer multiple comparisons). Uninoculated plants did not exhibit symptoms of FHB or mycotoxin accumulation; thus, their data are not shown.

2004) or three (in 2005) pots (replicates) for each timing of inoculation and each cultivar were tested in a completely randomized design. The spikes were spray inoculated with the inoculum at ≈ 20 ml/pot at the respective developmental stages between 1500 and 1800 h each day, and then were incubated overnight in a greenhouse at 18 to 25°C and 95 to 100% humidity provided by humidifiers. After inoculation, the plants were placed in a greenhouse (18 to 25°C) equipped with a sprinkler system that produced a fine mist intermittently to keep the spikes moist during the test. The severity of FHB was assessed 2 weeks after inoculation on a scale of 0 to 100 (0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100) according to visual rating of the percentage of infected florets. The evaluation was based on the average severity of typical diseased spikes in the pots; late or small spikes were ignored.

Effect of timing of infection on mycotoxin accumulation and FHB severity in barley cultivars (experiment 2). The experiment was conducted in Koshi (32.5°N, 130.4°E), Kumamoto, Japan, in 2004 and 2005. Five barley cultivars differing in row type and flowering type (Fig. 2) were used to examine the effect of the timing of *F. graminearum* infection on mycotoxin accumulation and the severity of FHB. ‘Russia 6’ was previously reported as highly resistant to FHB (36), and ‘Minorimugi’ is one of the leading barley cultivars in Japan. The other three cultivars also were tested in experiment 1.

The cultivars were seeded in 18-cm-diameter plastic pots (six plants per pot) at the beginning of December 2003 and 2004, and grown in a greenhouse under natural light until inoculation (April to May 2004 and 2005). The potting soil was prepared by mixing 30 liters of volcanic ash soil, 25 liters of vermiculite, 5 liters of

compost, 4 liters of leaf mold, and 50 ml of calcium magnesium carbonate. Each pot contained ≈ 2 liters of mixed soil and was fertilized with 2.5 g of $N-P_2O_5-K_2O$ at 8-8-8, 2 g of fused magnesium phosphate before seeding, and 1.4 g of $N-P_2O_5-K_2O$ at 8-8-8 at the stem elongation stage (ZGS 30 to 31). Late tillers of each plant were removed at the heading stage, leaving 7 to 12 similar-stage spikes per pot.

A mixture of two isolates of *F. graminearum* differing in trichothecene chemotype (i.e., H-3 of the DON chemotype and NIV-2 of the NIV chemotype) was used as the inoculum for experiment 2. Macroconidia of these isolates were produced as in experiment 1. The macroconidia concentration was provisionally adjusted to 2×10^6 spores/ml and then stored at -20°C until use. On the days of inoculation, the macroconidia suspensions of the two isolates were thawed and diluted to 5×10^5 spores/ml and then mixed in equal volumes. Finally, Tween 20 was added to a final concentration of 0.2 ml/liter.

Inoculation was performed at three developmental stages: anthesis (0 DAA), 10 DAA, and 20 DAA, with the exception of cv. Nishinochikara in 2005, which was inoculated at seven developmental stages: 2 days before anthesis and 3, 6, and 8 DAA, in addition to the above three stages. For each pot, 0 DAA was defined as the day on which all spikes in the pot reached anthesis. Because late tillers were removed at the heading stage, the spikes in each pot flowered almost uniformly. The developmental stages at 10 and 20 DAA corresponded to the early-milk (ZGS 73) and late-milk (ZGS 77) stages, respectively. Three pots (replicates) for each timing of inoculation for each cultivar were tested in a completely randomized design. For each cultivar, a control consisting of three pots of uninoculated plants also was prepared.

Fig. 3. Extrusion of the spent anthers in the cleistogamous barley cv. Nishinochikara. Spikes at **A**, 2 days after anthesis (DAA); **B**, 8 DAA; and **C**, 10 DAA. Appearance of the ventral side of florets at **D**, 6 DAA and **E**, 9 DAA. Anthers were still contained within the closed florets at 6 DAA; most anthers were partially extruded at 8 DAA; most anthers were completely extruded at 10 DAA. Arrows indicate the extruded spent anthers.

Inoculation was performed between 1400 and 1600 h on each designated day. Spikes of the potted plants were spray inoculated with the inoculum at 20 ml/pot using an airbrush (Piece Com PC-WIDE 308; Olympos, Nabari, Mie, Japan). Thereafter, the inoculated plants were maintained for 6 days under natural light in a greenhouse equipped with a sprinkler system that intermittently produced a fine mist to keep the inoculated spikes moist. The plants then were returned to the original greenhouse in which they were grown. The mean temperatures in the second and original greenhouses during the test period were 18.4 and 20.6°C in 2004 and 19.7 and 21.9°C in 2005, respectively. At 20 DAA, the severity of FHB was assessed. All spikes in each pot were evaluated individually on a scale of 0 to 100, as in experiment 1., and pot averages were calculated.

The spikes in each pot were harvested at maturity (32 to 35 DAA) and dried at 70°C for 24 h. After drying, the spikes were threshed carefully so that no small or light kernels were lost. The percentage of discolored kernels (DK), which showed tan to dark brown discoloration, was determined for each grain sample. DK were classified into two groups according to the proportion of discolored area, where DK1 = >50% of the grain surface was discolored and DK2 = 10 to 50% of the grain surface was discolored. After the evaluation of DK, the DON and NIV contents of each grain sample were analyzed. The grain samples were analyzed for mycotoxins using the previously described enzyme-linked immunosorbent assay system (45) by the KM Assay Center, Kyowa Medex Company Limited (Nagaizumi-cho, Shizuoka, Japan).

Statistical analyses. Statistical analyses were performed using Statview (version J5.0; SAS Institute, Cary, NC). For experiment 1, evaluation of the differences in FHB resistance at the two developmental stages for each cultivar, differences in the mean severity of FHB were tested using a *t* test for each year. For experiment 2, evaluation of the effect of the timing of infection on the mycotoxin levels in the mature grain, we compared levels within cultivars using Tukey-Kramer multiple comparisons for each year.

RESULTS

Evaluation of FHB resistance at two developmental stages in barley cultivars (experiment 1). In the cleistogamous cultivars, anthers did not protrude at anthesis; however, as the grain developed after pollination, the spent anthers were extruded from the tip of the floret between the tip of the palea and lemma (Fig. 3). Spent anther extrusion began at ≈7 DAA and was completed by 10 DAA.

When inoculated at anthesis, the tested cultivars showed a wide range in the severity of FHB in both years (Fig. 1). The level of disease severity caused by inoculation at anthesis differed depending on the flowering type: cleistogamous cultivars showed good FHB resistance, with severity of 15.0 to 20.0 in 2004 and 3.3 to 13.3 in 2005, whereas chasmogamous cultivars showed relatively high severity of FHB, regardless of row type, with FHB scores of 46.3 to 71.7 in 2004 and 50.0 to 60.0 in 2005. When inoculated at 10 DAA, however, the cultivars' responses differed from those obtained by inoculation at anthesis: cleistogamous cultivars were severely diseased, with severity of 40.0 to 68.8 in 2004 and 61.7 to 73.3 in 2005, as were chasmogamous cultivars, with FHB scores of 40.0 to 57.5 in 2004 and 40.0 to 70.0 in 2005.

A difference in resistance between the two developmental stages occurred in cleistogamous cultivars in both years. The severity of FHB following inoculation at 10 DAA was significantly higher than with inoculation at anthesis for each cultivar, with the exception of Nishinochikara in 2004 ($P = 0.11$). In cultivars inoculated at 10 DAA, symptoms of FHB, a brown discoloration, usually appeared around the tips of florets, where the spent anthers were extruded, after which the discoloration progressed toward the

bases of the florets. In contrast, in chasmogamous cultivars, the severity of FHB did not differ between inoculations done at 0 and 10 DAA, or the severity was higher in the former, although no differences were observed consistently in both years.

Effect of the timing of infection on mycotoxin accumulation and FHB severity in barley cultivars (experiment 2). In this experiment, the severity of FHB was assessed at 20 DAA for all plants, which is the commonly used timing for FHB evaluation in Japan; therefore, the severity of FHB in plants inoculated at 20 DAA was zero for all five cultivars. Following 20 DAA, the spike color gradually faded and turned brownish due to natural senescence, irrespective of cultivar, masking the appearance of the disease symptoms. As in experiment 1, anther extrusion in cleistogamous cultivars began at ≈7 DAA and was completed by 10 DAA.

The effect of the timing of infection on both mycotoxin accumulation and the severity of FHB differed between the two-rowed cleistogamous cultivars and the six-rowed chasmogamous cultivars (Fig. 2). Chasmogamous cultivars exhibited the highest severity of FHB when inoculated at 0 DAA. In contrast, cleistogamous cultivars were only slightly diseased when inoculated at 0 DAA and exhibited the highest severity of FHB when inoculated at 10 DAA, with the exception of Russia 6 in 2004, which had few symptoms when inoculated at 10 DAA. Thus, the timing of infection affected the severity of FHB differentially among cultivars that differed in flowering type. This is consistent with the results of experiment 1.

The total concentration of DON and NIV was highest with inoculation at 0 DAA for chasmogamous cultivars, similar to the severity of FHB. In contrast, the mycotoxin concentration in cleistogamous cultivars was significantly higher with inoculation at 10 DAA compared with 0 DAA, with the exception of Russia 6 in 2004, in which little mycotoxin accumulated when inoculated at 10 DAA (Fig. 2).

Data for Nishinochikara in 2005 indicated that the change in this cultivar's response to inoculation between 0 and 10 DAA actually occurred between 6 and 10 DAA because the severity of FHB and mycotoxin concentration with inoculation at 10 DAA were significantly higher than with inoculation at 6 DAA and earlier. At 6 DAA, all anthers were still inside the closed florets; by 8 DAA, most anthers had been partially extruded; at 10 DAA, most anthers had been completely extruded (Fig. 3).

Inoculation at 20 DAA did not cause clear symptoms of FHB in any cultivar. However, it did result in accumulation of DON and NIV. The cleistogamous cultivars, in particular, except for Russia 6, accumulated high levels of DON and NIV with inoculation at 20 DAA (DON + NIV: 9.0 to 58.8 µg/g), and the total concentration of DON and NIV in these cultivars was significantly higher than when inoculated at 0 DAA. The chasmogamous cv. Minorigumi also accumulated high levels of DON and NIV after inoculation at 20 DAA (DON + NIV: 10.1 and 10.7 µg/g in 2004 and 2005, respectively); however, this concentration was lower than that with inoculation at 0 and 10 DAA, although the difference did not reach significance in 2004 ($P = 0.059$ by analysis of variance). For chasmogamous cultivars, both the severity of FHB and mycotoxin concentrations declined when infection timing was delayed.

The change in the percentage of DK with the later timing of infection (Fig. 4) was associated with the change in the severity of FHB (Fig. 2), except in cvs. Nishinochikara and Daisen Gold in 2005. These two cultivars did not show distinct symptoms of FHB on the spikes following inoculation at 20 DAA in either year (Fig. 2), although the harvested grain samples in 2005 contained 34.5 and 49.6% DK, respectively; however, most of these were stained only around the base of the kernel and were graded as DK2 (10 to 50% of the grain surface discolored) (Fig. 4). For all cultivars, the change in the percentage of DK in 2005 was similar to the pattern of mycotoxin accumulation, rather than the severity of FHB (Figs. 2 and 4).

Unlike in 2005, the change in the percentage of DK in 2004 was associated with the change in the severity of FHB. The kernels from plants inoculated at 20 DAA accumulated high levels of mycotoxin without clear disease symptoms being present (Figs. 2 and 4). The cvs. Nishinochikara and Minorimugi accumulated mycotoxin (mean DON + NIV) at 9.0 and 10.1 µg/g, respectively, following inoculation at 20 DAA in 2004, without displaying clear disease symptoms on the spike or the kernels (Figs. 2, 4, and 5).

DISCUSSION

To our knowledge, this is the first investigation of the effect of the timing of *Fusarium* infection on mycotoxin accumulation and severity of FHB in barley. We investigated the accumulation of both DON and NIV because they can often co-occur (25,37, 38,43,44).

The timing of inoculation had different effects on the severity of FHB between the two barley flowering types. Cleistogamous cultivars exhibited good resistance when inoculated at anthesis, but susceptibility increased drastically with inoculation at 10 DAA. In contrast, chasmogamous cultivars were susceptible to inoculation at anthesis, and this susceptibility was retained or de-

clined at 10 DAA. These results were similar in two separate experiments. Furthermore, in experiment 2, the mycotoxin concentration in harvested grain was affected by the timing of infection in a manner similar to the severity of FHB with inoculations between 0 and 10 DAA; the mycotoxin concentration in the two-rowed cleistogamous cultivars was much higher with inoculation at 10 than at 0 DAA in most cases. Although we tested only five cultivars for mycotoxin accumulation, this increase in accumulation of mycotoxins associated with susceptibility to FHB after anthesis likely is common in two-rowed cleistogamous cultivars, because an increase in susceptibility to FHB occurred in all two-rowed cleistogamous cultivars tested in experiment 1.

The drastic increase in susceptibility to FHB and mycotoxin accumulation in the two-rowed cleistogamous cultivars appears attributable to the extrusion of the spent anthers, which occurs after anthesis, because (i) previous studies with wheat have shown that initial *Fusarium* infection occurs on fully or partially extruded anthers (26) and that anther extracts enhance fungal growth (5,33), suggesting that anthers may play a fundamental role in infection by *F. graminearum* and other *Fusarium* spp. (26,33); (ii) recent studies of near-isogenic barley lines indicate that cleistogamy contributes to resistance to FHB at anthesis (42), suggesting

Fig. 4. Percentage of discolored kernels (DK) for five barley cultivars and different inoculation times with *Fusarium graminearum* in 2004 and 2005. Numbers below the graph indicate the timing of inoculation in days after anthesis (DAA). Each point represents the mean of three replicates; bars represent standard errors.

that the extrusion of the anthers in barley also may promote infection by *F. graminearum*; (iii) in cleistogamous cultivars inoculated at 10 DAA, symptoms of FHB usually appeared around the tips of the florets, where the spent anthers extruded and usually remained; and (iv) the increase in susceptibility to FHB in cv. Nishinochikara in 2005 occurred between 6 and 10 DAA, during which time the extrusion of the anthers actually occurred. It is likely that the extruded spent anthers provide an initial base for the colonization of *F. graminearum* in resistant cleistogamous cultivars.

Lewandowski et al. (14) recently reported that anthers may be less important for infection by *F. graminearum* in barley than in wheat, based on their field inoculation experiments using the susceptible six-rowed cv. Robust. In their study, florets with colonized anthers were not observed frequently following inoculation, although some anthers were colonized and provided a bridge for fungal entry into florets, as previously observed in wheat (26). It is possible that the importance of the anthers in infection by *F. graminearum* differs with barley cultivars that differ in resistance or flowering type. Further studies are needed to elucidate the infection pathway at different developmental stages in resistant cleistogamous cultivars, as well as in fully susceptible cultivars.

Barley usually undergoes anthesis after heading (2). However, in some regions where barley is grown, anthesis occurs while the spike is still enclosed within the flag leaf sheath (16,32). The experiments by Lewandowski et al. (14) cited above were performed in the Upper Midwest region of the United States (14), where barley plants commonly flower before heading (32). It is not described when flowering actually occurred in their study; however, if it occurred before heading, this also may have caused the apparent inconsistency between their and our studies. In the situations where anthesis occurs before heading, the importance of the anthers in infection by *F. graminearum* and the possible contribution of flowering type and anther extrusion to changes in

resistance to FHB after anthesis might differ from that described in the present study. Nevertheless, the resistance to FHB may be greatly affected by the timing of anther extrusion when this occurs after heading.

The accumulation of DON and NIV in the grain resulted from infection events up to 20 DAA, even when such late infection did not display distinct symptoms of FHB. Thus, even if the severity of FHB assessed in the field appears absent or very low, DON and NIV can accumulate in the mature grain as a result of late infections. In addition, the mycotoxin levels found are not necessarily low. This risk appears to be barley cultivar dependent, but is not related to the flowering type or row type. Among the tested cultivars, Nishinochikara, Daisen Gold, and Minorimugi appear to be at high risk of mycotoxin contamination following late infection, whereas the cvs. Russia 6 and Chevron appear to be at low risk. For such late infection, infection courts other than extruded anther may play an important role. There may exist resistance mechanisms for such late infection which are unrelated to spike morphology and independent of resistance at early stages. Russia 6 and Chevron may possess such "late resistance." Remarkably, the Japanese two-rowed cleistogamous cvs. Nishinochikara and Daisen Gold accumulated comparable levels of mycotoxins with inoculation at 10 and 20 DAA, and these levels were much higher than those accumulated following inoculation at anthesis. Thus, late infection without visible symptoms of FHB cannot be ignored in terms of there being a risk of mycotoxin contamination.

Furthermore, high levels of DON and NIV contamination in barley grain resulting from late infection may even lack visible symptoms on the grain. The cvs. Nishinochikara and Minorimugi, which accumulated high levels of mycotoxin when inoculated at 20 DAA, exhibited some discoloration of the grain in 2005 but not in 2004. It is not clear what caused this difference between years; however, minor differences in environmental conditions or other microorganisms that also cause kernel discoloration in barley (20) may have been involved.

Fig. 5. Appearance of mature grains from barley cultivars **A**, Nishinochikara and **B**, Minorimugi with and without inoculation with *Fusarium graminearum* in 2004. Treatments: **a**, uninoculated; **b**, inoculated at anthesis; **c**, inoculated at 10 days after anthesis (DAA); **d**, inoculated at 20 DAA. Cv. Nishinochikara samples **a**, **b**, **c**, and **d** contained mycotoxin (deoxynivalenol plus nivalenol) at 0, 0.4, 11.8, and 9.0 $\mu\text{g/g}$, respectively; Cv. Minorimugi samples **a**, **b**, **c**, and **d** contained mycotoxin at 0, 70.9, 27.0, and 10.3 $\mu\text{g/g}$, respectively.

The relationship between mycotoxin concentrations and visual symptoms of FHB on spikes and grain is weak or inconsistent in both barley (3,29,39) and wheat (15,19,23,24,31). For barley, this can be attributed in part to the presence of other pathogens that cause symptoms similar to those of FHB (3,29). In addition, the present study demonstrated that differences in the timing of infection also can contribute to inconsistencies in the relationship between mycotoxin concentration levels and disease severity, in both the spike and grain. Barley kernels that are normal in appearance may contain high levels of DON (32,39). Our data suggest that such cases may be caused by late infection.

There currently are no robust control measures to manage either FHB or mycotoxin contamination in barley and wheat. Fungicide application is one measure available to reduce the risk; however, this has not been highly effective or consistent in barley or wheat (8,11,18,32). Although studies of chemical control of FHB in barley are limited, a fungicide generally is applied at or near anthesis (6,10,11), similar to its application in wheat. In Japan, fungicide application for control of FHB in barley usually is performed around the time of anthesis, which occurs a few days after the full heading stage. Thus, the time of anthesis is regarded as the most important stage for *Fusarium* infection in barley, probably following the example of wheat. However, we have demonstrated that the most critical time for *F. graminearum* infection and subsequent mycotoxin accumulation in barley differs among cultivars and appears to be associated with the flowering type. For two-rowed cleistogamous cultivars in Japan, infection that occurs after the extrusion of spent anthers would be much more important than infection around anthesis. Our findings suggest that the optimal timing for chemical control of FHB and mycotoxin contamination in barley will depend on the cultivar. For cleistogamous cultivars, the efficacy of fungicide applications is expected to be improved by changing the timing of application to near the extrusion of spent anthers. Fungicide trials to test this prediction are currently underway.

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