Effect of the Timing of Fungicide Application on Fusarium Head Blight and Mycotoxin Accumulation in Closed-Flowering Barley

Megumi Yoshida, Takashi Nakajima, Michiyoshi Arai, Fumihiko Suzuki, and Kenta Tomimura, Research Team for Fusarium Head Blight Control, National Agricultural Research Center for Kyushu Okinawa Region (KONARC), 2421 Suya, Koshi, Kumamoto 861-1192, Japan

ABSTRACT

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Fungicide application is one measure available to reduce the risk of Fusarium head blight (FHB) and mycotoxin contamination in barley. The stage at or near anthesis, or at full head emergence, is generally thought to be optimal for fungicide application, regardless of cultivar. However, we have previously found that the most critical time for *Fusarium graminearum* infection and mycotoxin accumulation in barley differs among cultivars. Whereas chasmogamous (open-flowering) cultivars were most susceptible at anthesis, cleistogamous (closed-flowering) cultivars were considerably resistant at anthesis but became susceptible after 'spent' anther extrusion. Therefore, this study evaluated the effect of the timing of fungicide application on FHB and mycotoxin (deoxynivalenol and nivalenol) accumulation in cleistogamous barley. Thiophanate-methyl fungicide was applied at different developmental stages, from before anthesis to 30 days after anthesis (DAA), under artificial inoculation conditions in the field in which inoculum spores were provided throughout the testing period. As expected, the optimal timing for chemical control of FHB and mycotoxin accumulation was the time around the beginning of spent anther extrusion rather than at anthesis. Later application, as late as 30 DAA, was also effective in controlling mycotoxin accumulation, although it was not effective in controlling disease levels.

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

Fusarium head blight (FHB) or scab, which is caused by several *Fusarium* spp., especially *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), is a widespread and destructive disease of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (25,32,38). 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. The contamination of barley and wheat by these mycotoxins has been reported worldwide (34,44,45).

F. graminearum can be divided into two chemotaxonomic groups: the DON chemotype and the NIV chemotype (14,20,37,39).

DON is the most prevalent mycotoxin in cereals (1,34), and DON chemotypes of *Fusarium* spp. prevail worldwide. NIV chemotypes are more restricted (18,29); therefore, more attention is generally 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 in 2001 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA; 1). However, NIV is also an important *Fusarium* mycotoxin that frequently occurs worldwide (34,44) and commonly co-occurs with DON (34,44,45,51,52). In Japan, where *F. graminearum* is the main causal organism of FHB (19), the isolation frequency of NIV chemotypes is greater than that of DON chemotypes in some areas (13,30,41,52). More importantly, NIV is more toxic to animals than DON (36,42,47). Accordingly, control measures that are effective for both DON and NIV should be established.

There is currently no robust single control measure by which to manage either FHB or mycotoxin contamination in barley and wheat. Fungicide application is one measure available to reduce the risk; however, results have not been highly effective or consistent (12,16,25,26,33,38). To ob-

tain increased chemical control of FHB, the timing of fungicide application is an important factor, as well as fungicide selection, application rate, and good coverage of the spike (26).

For wheat, anthesis is regarded as the optimal growth stage for fungicide application to control FHB (26,33). This is reasonable because wheat is most susceptible to FHB during anthesis (4,32,35,40), at which time anthers extrude from the florets. Initial *Fusarium* spp. infection commonly occurs on extruded anthers (35).

For barley, in most studies of chemical control of FHB, fungicides were applied at or near anthesis (10,15,48), similar to the case for wheat, or at full head emergence (10,16,27), which can be either concurrent with or different from the time of anthesis. Although anthesis in barley usually occurs after heading (5), in some barley-growing regions such as major barley production areas of North America, anthesis occurs before heading (22,38). In such cases, the time of full head emergence should be after anthesis is completed. In Japan, where anthesis in barley usually occurs at or a few days after full heading, fungicide application for FHB has been performed around anthesis regardless of cultivar; however, this practice is not based on appropriate research evidence.

Whereas wheat is generally chasmogamous (open-flowering type) and extrudes anthers at anthesis, barley has two flowering types: chasmogamous and cleistogamous (closed-flowering type). In cleistogamous cultivars, the florets do not open and anthers are not extruded at anthesis. However, the anthers may be pushed out from the tips of florets by the developing grain several days after anthesis (5). In Japan, this phenomenon is observed commonly in cleistogamous cultivars. Thus, the timing of anther extrusion differs with flowering type in barley.

For Japanese barley cultivars, there is a strong association among row type, flowering type, and FHB resistance. Most two-rowed cultivars are cleistogamous and resistant, whereas most six-rowed cultivars are chasmogamous and susceptible (50). These associations are consistent with the general observations that two-row types are more resistant than six-row types (7,11,23,38,43,55). Cleistogamy, rather than row type, contributes to the resistance of barley cultivars to FHB, at least for

Corresponding author: T. Nakajima
E-mail: ntakashi@affrc.go.jp

Current address of Kenta Tomimura: Kuchinotsu Citrus Research Station, National Institute of Fruit Tree Science, Kuchinotsu, Nagasaki 859-2501, Japan.

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infection that occurs at anthesis (50). However, even in two-row cleistogamous cultivars with relative resistance, high levels of DON and NIV contamination can occur (49).

We previously found that the most critical time for *F. graminearum* infection and mycotoxin accumulation in barley differs between open- and closed-flowering types (49). Whereas chasmogamous cultivars were found to be most susceptible at anthesis, cleistogamous cultivars were considerably resistant against FHB at anthesis but became susceptible shortly after the extrusion of 'spent' anthers (49). This was probably because the extruded spent anthers provide an initial base for the colonization of *F. graminearum* in cleistogamous cultivars. Thus, it seems likely that the optimal timing for chemical control of FHB and mycotoxin contamination in barley will differ with flowering type. For cleistogamous cultivars, we expect that the efficacy of fungicide application will be improved by changing the application timing from anthesis to the extrusion of spent anthers.

To date, only a few studies have been published on the effect of the timing of fungicide application in controlling FHB and mycotoxin accumulation in barley. McMullen et al. (24) noted that early studies in North Dakota indicated that the optimum time for fungicide application on barley to control FHB is shortly after head emergence. In addition, preliminary experimental results by the authors (24) and Jordahl et al. (17) appeared to show that fungicide application controlled FHB better when applied at half or full head emergence than when applied at the 'kernel watery ripe' stage. As for controlling the mycotoxin, Neate et al. (31) reported in their preliminary results that split applications of fungicide (at awns emerged and at full head emergence) did not provide a significant advantage over a single application to control FHB and DON under low disease pressure. All of the reports were,

however, from the Upper Midwest region of the United States, where anthesis in barley usually occurs before heading, and the flowering types of the tested cultivars were not noted in the reports. In addition, tested application timings were limited, and mycotoxin data were lacking in most cases.

The objective of this study was to determine the optimal timing of fungicide application for controlling FHB and mycotoxin (DON and NIV) accumulation in cleistogamous barley.

MATERIALS AND METHODS

Plant materials. The effects of the timing of fungicide application on cleistogamous barley were evaluated during 2005 and 2006. The experiments were performed in the field at the National Agricultural Research Center for Kyushu Okinawa Region (KONARC), in Koshi (32.5° N, 130.4° E), Kumamoto, Japan. The two-row cleistogamous cv. Nishinochikara, which is one of the most widely grown cultivars in Japan, was used in both years. Nishinochikara is relatively resistant to FHB, as are most two-rowed cleistogamous cultivars in Japan (49,50). This cultivar was planted on 25 and 24 November in 2004 and 2005, respectively, in single rows that were 0.8 m apart and 50 m long. The plants were grown in accordance with standard agronomic practices until heading.

Inoculation and spore monitoring. Inoculation with *F. graminearum* was performed using colonized maize kernel inoculum (6,8), which generates ascospores over a long period during the testing season in the field. A mixture of four Japanese isolates of *F. graminearum* differing in trichothecene chemotype (i.e., H-3 and DON-5 of the DON chemotype and NIV-1 and NIV-7 of the NIV chemotype) was used. Colonized maize kernels of each isolate were prepared as follows. One liter of maize kernels was placed into 2-liter Erlenmeyer flasks, without adding water, and the flasks were covered with aluminum foil and autoclaved once at 121°C for

1 h. Macroconidia of each *F. graminearum* isolate were produced as in our previous study (49) using mung bean liquid medium, and the macroconidial suspension of each isolate (1 to 4 × 10⁶ conidia/ml) was diluted 10 times with sterile distilled water. The diluted macroconidial suspension was then injected into flasks (110 ml of suspension per flask) and shaken to ensure distribution throughout the maize kernels. The flasks were maintained in an incubator without light at 25°C for 2 weeks to permit thorough colonization of the kernels. During incubation, the flasks were periodically shaken. After 2 weeks of incubation, the colonized maize kernels were spread in stainless vats and naturally dried for 3 days. The dried kernels were stored at -20°C until use (approximately 2 months). On 24 March 2005 and 27 March 2006, at the early-boot stage (Zadoks growth stage [ZGS] 41), the colonized maize kernels of the four isolates were mixed in equal volumes and distributed in the field by hand at rates of 48 and 43 ml/m², respectively. In total, approximately 24 and 21.5 liters of colonized maize kernels were distributed in the field (approximately 500 m²) in 2005 and 2006, respectively.

Mist irrigation to promote ascospore production and fungal infection was performed in the field using a sprinkler system with DN881A-type sprinkler heads equipped with 1.50-mm-diameter nozzles (Sun Hope Inc., Meguro-ku, Tokyo). The sprinkler system was set up before heading, spacing the sprinkler heads approximately 2 m apart, and was activated on 9 April (ZGS 49; first awns visible) in 2005 and 16 April (ZGS 56 to 57; three-quarters of the inflorescence emerged in >50% of spikes) in 2006. The start of irrigation was delayed in 2006 because there was frequent natural precipitation from the beginning of April in that year. Water was applied four times daily (at 0100, 0700, 1300, and 1900 h) for 15 min at each time in 2005 and for 6 min at each time in 2006, at a rate of approximately 25 mm/h. On

Table 1. Timing of fungicide application to closed-flowering barley (cv. Nishinochikara) grown in the test field in Koshi Japan in 2005 and 2006^a

Code	Plant growth stage description	ZGS ^b	2005		2006	
			DAA	Date	DAA	Date
I	Before anthesis	56 to 57	-3	15 April	-2	16 April
II	Anthesis	(59) 64 to 65	0	18 April	0	18 April
III-1	4 to 5 DAA	71	4	22 April	5	23 April
III-2	9 DAA, before spent anther extrusion	...	9	27 April	9	27 April
IV-1	Beginning of spent anther extrusion ^c	73	11	29 April	12	30 April
IV-2	Spent anther extrusion half-way ^d	...	...	...	14	2 May
IV-3	Spent anther extrusion complete ^e	75	15	3 May	16	4 May
V	Late milk	77	21	9 May	21	9 May
VI	Soft dough	85	29	17 May	30	18 May

^a DAA = days after anthesis.

^b Zadoks growth stage (ZGS) corresponded to each timing. ZGS 56 to 57: three-quarters of the inflorescence emerged in >50% of spikes; ZGS 59: emergence of inflorescence completed; ZGS 64 to 65: anthesis half-way in >50% of spikes; ZGS 71: caryopsis water ripe; ZGS 73: early milk; ZGS 75: medium milk; ZGS 77: late milk; ZGS 85: soft dough.

^c Spent anther tips emerged in 50% of spikes.

^d Spent anthers fully extruded in 50% of spikes.

^e Spent anthers fully extruded in >90% of spikes.

rainy days, irrigation was not performed. Misting was continued until 4 and 6 days before harvest in 2005 and 2006, respectively. Daily mean temperature and precipitation amount during the test period were recorded at the meteorological station at KONARC, located approximately 750 m north of the test field.

The daily inoculum spore load (*F. graminearum* spores) during the test period was monitored at 1- to 5-day intervals using the selective medium (FG medium) developed by Togawa (46) for the isolation of *F. graminearum*. Before heading, a plate stand (80 by 20 by 20 cm high) with a roof (180 by 70 by 80 cm high) was set up in

the center of the field. On each monitoring day, three plates of FG medium (in 9-cm-diameter plastic petri dishes) were placed on the stand in a row at a spacing of 10 cm between 1630 and 1730 h each day, and they were retrieved on the next day between 0830 and 0930 h. The plates were covered with lids, sealed with Parafilm, and incubated under fluorescent light at room temperature ($23 \pm 2^\circ\text{C}$). After approximately 1 week, *F. graminearum* colonies, identified as having white aerial hyphae on the surface and a bright red color from the underside of plates, were counted, and the average of three plates was calculated.

Experimental design and fungicide treatments. The timings of fungicide application (Table 1) were represented by codes from I to VI, which were arranged by the authors. This is because published growth stage scales such as ZGS (54) and Feekes growth stage (9) cannot appropriately describe the growth stages shortly before or during spent anther extrusion, which were tested in this study.

Treatments (different timings of fungicide application and no-fungicide control) were assigned to 4-m single-row plots that were arranged at 0.5-m intervals in a randomized complete block design in the field consisted of 12 50-m-long rows spaced 0.8 m apart. Adjacent rows were left untreated as border rows. Each treatment was replicated three and four times in 2005 and 2006, respectively.

A wettable powder of Topsin M (Nippon Soda Co., Ltd. Chiyoda-ku, Tokyo), containing 70% thiophanate-methyl as the active ingredient (a.i.), was used as the fungicide treatment. Topsin M is one of the most widely used fungicides to control FHB in barley and wheat in Japan (2,3). This product was diluted in water at 1 g liter⁻¹ and a spreader, New Gramin (Sankyo Agro Co., Ltd. Minato-ku, Tokyo), was added at 0.3 ml liter⁻¹; the mixture was applied at a rate of 150 ml/m² using manual pressure sprayers with 1.2-liter capacity (T-1; Sakata Seed Co., Yokohama, Japan). This was equivalent to 1,050 g a.i./ha. Fungicide application was performed between 1300 and 1500 h on each designated day. Sprinkler irrigation at 1300 h was not performed on the days of fungicide application.

Disease and post-harvest assessments. FHB was assessed at 22 and 24 days after anthesis (DAA) in 2005 and 2006, respectively (ZGS 77; late-milk stage). Fifty spikes were arbitrarily selected from each plot and assessed for FHB severity on a scale of 0 to 100 (0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100) according to a visual rating of the percentage of infected area in each spike, and the average was calculated for each plot. Using these data, percent FHB incidence (i.e., the percentage of visually diseased spikes) was also calculated for each plot.

Fig. 1. Daily inoculum spore load of *Fusarium graminearum* trapped on *F. graminearum* selective medium, daily mean temperature, and daily precipitation during the test period in each study year. Broken arrows indicate periods of sprinkler irrigation, performed four times a day. The timings of fungicide application are indicated in bold, solid arrows with the stage codes I = before anthesis; II = anthesis; III-1 = 4 to 5 days after anthesis (DAA); III-2 = 9 DAA, before spent anther extrusion; IV-1 = beginning of spent anther extrusion; IV-2 = spent anther extrusion half-way; IV-3 = spent anther extrusion complete; V = late milk; and VI = soft dough. Bold, open arrows indicate the day of harvest.

At physiological maturity (ZGS 91, 38 and 41 DAA in 2005 and 2006, respectively), all spikes within 2 m of the middle of each single-row plot were harvested by hand. The spikes were dried at 70°C for 24 h and threshed with a thresher. The harvested grain samples were cleaned manually. The percentage of discolored kernels (DKs) showing tan to dark brown discoloration on >5% of the grain surface was determined for each grain sample. The thousand-kernel weight (TKW) was also measured for each grain sample. The DON and NIV contents were analyzed for 100 g of each grain sample using the previously described enzyme-linked immunosorbent assay (ELISA) system (53) of the KM Assay Center, Kyowa Medex Company Limited (Nagaizumi-cho, Shizuoka, Japan).

Statistical analyses. The effect of treatments on FHB incidence, FHB severity, TKW, DK percentage, and mycotoxin (DON, NIV, and DON+NIV) content was tested using analysis of variance (ANOVA) within each year, followed by Fisher's protected least significant difference (PLSD) test at a significance level of $P = 0.05$. The efficacy of each timing of fungicide application was calculated for each year using the formula $[(K - P)/K] \times 100$; where K is the mean of each assessed parameter (FHB incidence, FHB severity, DK percentage, and DON+NIV content) in the no-fungicide control plots and P is the mean of each parameter in the treated plots.

RESULTS

Spore monitoring and growth of plant materials. The numbers of *F. graminearum* spores trapped on the selective medium over the test period in each year are shown in Figure 1, together with the meteorological data. In both years, *F. graminearum* spores were observed on the first day of spore monitoring [42.0 CFU/plate on 9 April 2005 and 15.7 CFU/plate on 12 April 2006]. The number of *F. graminearum* spores trapped on the first day of fungicide application (stage I: before anthesis) was 32.0 and 35.3 CFU/plate in 2005 and 2006, respectively; after that, more spores were always trapped on each monitoring day until harvest (26 and 29 May in 2005 and 2006, respectively), except on 20 April 2006 (2 DAA, 8.7 CFU/plate). The daily number of *F. graminearum* spores was not correlated with natural precipitation, probably because of sprinkler irrigation during this period. Overall, the number of spores collected during the test season was greater in 2005 than in 2006. The maximum daily number of trapped spores was 12.0×10^2 CFU/plate in 2005 and 6.2×10^2 CFU/plate in 2006.

In both years, the barley plants reached head emergence (ZGS 52 to 53; one-quarter of the inflorescence emerged in >50% of spikes) on 14 April and reached anthesis (ZGS 64 to 65; anthesis half-way in >50% of spikes) on 18 April. On the day of anthesis, most spikes had also just completed emergence (ZGS 59).

Effect of each fungicide application timing on disease and post-harvest pa-

rameters. In both years, the effect of fungicide timing was significant (ANOVA; $P < 0.05$) for all disease and post-harvest parameters evaluated except TKW ($P = 0.10$ and 0.16 in 2005 and 2006, respectively). Disease level of the no-fungicide control plots was moderately severe in 2005 (FHB incidence = 98.0% and FHB severity = 13.7), whereas it was slight in 2006 (FHB incidence = 59.5% and FHB severity = 3.5). The mycotoxin content in grain from the control plots was also higher in 2005 (DON + NIV = 25.0 µg/g) than in 2006 (DON + NIV = 6.1 µg/g). The DK percentage, however, was numerically higher in 2006 (11.1%) than in 2005 (8.7%).

In both years, FHB incidence, FHB severity, and DK percentage were significantly lowered by fungicide application at stages III-1 to IV-3, and mycotoxin (DON and NIV) accumulation was significantly lowered by application at stages II to VI, except that the application at stage III-1 in 2006 did not significantly lower NIV accumulation (Table 2). Additionally, in 2005, FHB severity, DK, and mycotoxin (DON and NIV) levels were also lowered by application at earlier stages (stages I and II); however, such results were not obtained in 2006.

In 2005, the maximum control efficacy was obtained from fungicide application at plant growth stages III-2 to IV-1 for all of the parameters (FHB incidence, FHB severity, DK, and DON + NIV; Fig. 2), although the effects of fungicide application were not significantly different during III-1

Table 2. Effect of the timing of thiophanate-methyl fungicide application on Fusarium head blight (FHB) incidence (I), FHB severity (S), thousand-kernel weight (TKW), percentage of discolored kernels (DK), and deoxynivalenol (DON) and nivalenol (NIV) concentration in closed-flowering barley (cv. Nishinohikara) in each study year^a

Year, treatment ^b	FHB I (%)	FHB S (0-100)	TKW (g)	DK (%)	DON (µg/g)	NIV (µg/g)	DON+NIV (µg/g)
2005							
I	90.7 a	9.1 c	35.0	4.0 b	10.5 bc	2.0 b	12.5 bc
II	58.7 ab	9.2 c	33.5	4.7 b	11.3 b	2.1 b	13.4 b
III-1	46.0 bc	3.5 d	36.8	1.8 c	7.0 bc	1.1 bc	8.1 bc
III-2	18.0 c	1.7 d	37.5	1.5 c	5.3 bc	0.7 c	6.0 bc
IV-1	22.0 bc	1.6 d	37.3	1.4 c	3.8 c	0.6 c	4.4 c
IV-3	48.7 bc	3.5 d	34.6	3.0 bc	6.4 bc	1.2 bc	7.6 bc
V	96.7 a	10.7 bc	33.1	7.3 a	10.7 bc	1.9 bc	12.6 bc
VI	98.7 a	14.9 a	34.0	7.6 a	12.1 b	2.3 b	14.4 b
No fungicide	98.0 a	13.7 ab	31.8	8.7 a	21.3 a	3.6 a	25.0 a
LSD ($P = 0.05$)	40.0	3.1	ns	1.8	7.5	1.2	8.5
2006							
I	59.0 a	3.3 ab	33.3	9.3 ab	3.9 ab	1.2 ab	5.1 ab
II	59.0 a	3.3 ab	34.5	8.8 ab	2.6 bc	1.0 b	3.6 bc
III-1	41.5 c	2.2 cd	34.6	7.0 bc	3.1 bc	1.2 ab	4.3 abc
III-2	35.0 c	2.0 cd	35.4	6.0 c	2.0 cd	0.9 bc	2.9 cd
IV-1	34.5 c	1.8 d	35.8	5.1 c	1.0 d	0.5 cd	1.5 d
IV-2	41.0 c	2.2 cd	34.8	5.2 c	0.9 d	0.3 d	1.2 d
IV-3	43.0 bc	2.7 bc	35.4	6.2 c	0.9 d	0.3 d	1.3 d
V	52.5 ab	3.1 ab	33.7	8.7 ab	2.1 cd	0.9 bc	2.9 cd
VI	57.0 a	3.4 a	33.5	9.4 ab	2.0 cd	0.7 bcd	2.8 cd
No fungicide	59.5 a	3.5 a	34.3	11.1 a	4.6 a	1.5 a	6.1 a
LSD ($P = 0.05$)	10.3	0.8	ns	2.4	1.4	0.5	1.9

^a Values indicate the mean of three or four replications per treatment. Values within a year and column followed the same letter are not significantly different according to Fisher's protected least significant difference (LSD) test at $P = 0.05$; ns = not significant.

^b Timing of fungicide application is indicated in the stage codes: I = before anthesis; II = anthesis; III-1 = 4 to 5 days after anthesis (DAA); III-2 = 9 DAA, before spent anther extrusion; IV-1 = beginning of spent anther extrusion; IV-2 = spent anther extrusion half-way; IV-3 = spent anther extrusion complete; V = late milk; and VI = soft dough.

to IV-3 for disease parameters or during III-1 to V for DON and NIV (Table 2). The maximum efficacy levels for each parameter in 2005 were similar (Fig. 2): the efficacy values at stages III-2 and IV-1 were 81.6 and 77.6 for FHB incidence, 87.3 and 88.1 for FHB severity, 82.4 and 83.5 for DK, and 75.9 and 82.3 for DON + NIV.

In 2006, the maximum efficacy levels differed among the parameters (Fig. 2). For mycotoxin content (DON + NIV), the maximum efficacy was obtained with fungicide application at plant growth stages IV-1 to IV-3 and the efficacy values were

75.2 to 79.9, although the effects of fungicide application were not significantly different during stages III-2 to VI (Table 2). For disease parameters (FHB incidence, FHB severity, and DK), the maximum efficacy values were 42.0, 50.0, and 53.5, respectively. The maximum efficacy values were obtained with application at stage IV-1, although the effects of fungicide application on the disease parameters were not significantly different during stages III-1 to IV-2 (Table 2).

Thus, through the 2 years, the optimal effect of fungicide application was ob-

tained at plant growth stages III-1 (4 to 5 DAA) to VI-2 (spent anther extrusion half-way) for disease parameters (FHB incidence, FHB severity, and DK) and at stages III-2 (9 DAA) to V (late milk) for mycotoxin content (DON + NIV), and (numerically) maximal control efficacy was obtained by application around stage VI-1 (beginning of spent anther extrusion) for all the parameters.

In both years, fungicide application at late stages (i.e., V and VI, 21 and 29 to 30 DAA, respectively) did not significantly affect disease levels (FHB incidence, FHB severity, and DK); however, it did significantly lower grain mycotoxin content (DON and NIV) (Table 2). In 2005, the efficacy of application at stages V and VI in lowering the mycotoxin content (DON + NIV) was 49.7 and 42.4, respectively, and, in 2006, the efficacy was 52.0 and 54.7, respectively.

DISCUSSION

To our knowledge, this is the first study to intensively evaluate the effects of the timing of fungicide application on FHB and on DON and NIV mycotoxin accumulation in cleistogamous barley. The results obtained in this study suggest that the optimal time for chemical control of FHB and mycotoxin contamination in cleistogamous barley is the time around the beginning of spent anther extrusion, rather than at anthesis. In addition, fungicide application as late as 30 DAA may also be effective in controlling mycotoxin accumulation in grain.

In both test years, fungicide application around the beginning of spent anther extrusion resulted in the best efficacy for controlling FHB and mycotoxin content in a cleistogamous barley cultivar. The efficacy of fungicide application around the beginning of spent anther extrusion on the mycotoxin content was similarly high (approximately 80%) in both years. However, the efficacy of application at this stage, in terms of visual assessment of disease levels, appeared to differ between the years. The efficacy for each disease parameter in 2005 was approximately 80% but, in 2006, it was only approximately 50%. This may be attributed, at least in part, to the difference in disease levels between the years. Based on an analysis of data from over 100 wheat studies in the United States, Paul et al. (33) reported that the efficacy of fungicide (tebconazole) was apt to be low and the variability to be high when FHB or DON levels in control plots were low. These results may indicate that the efficacy of application at this stage on mycotoxin content is more consistent than that on disease parameters; however, further experiments are needed to confirm this.

These results, indicating a higher efficacy of fungicide application at around spent anther extrusion than at earlier stages, support the results of our previous

Fig. 2. Control efficacy at various times of thiophanate-methyl fungicide application on Fusarium head blight (FHB) incidence, FHB severity, percentage of discolored kernels (DK), and concentration of deoxynivalenol (DON) plus nivalenol (NIV) in each study year. The timing of fungicide application is indicated in the stage codes I = before anthesis; II = anthesis; III-1 = 4 to 5 days after anthesis (DAA); III-2 = 9 DAA, before spent anther extrusion; IV-1 = beginning of spent anther extrusion; IV-2 = spent anther extrusion half-way; IV-3 = spent anther extrusion complete; V = late milk; and VI = soft dough.

study (49), showing much higher susceptibility after spent anther extrusion than at early stages in cleistogamous barley. Changes in susceptibility of the host plant during the season likely had a major effect on these results. However, higher efficacy at spent anther extrusion may partly be attributed to the relatively low infection pressure (low daily temperature and low inoculum) during the early stages.

It is noteworthy that fungicide application at later stages (21 and 29 to 30 DAA, ZGS 77 and 85, respectively) resulted in significantly lower DON and NIV content compared with the no-fungicide control, although it did not significantly affect disease levels. Our previous study (49) showed that infection as late as 20 DAA could cause high levels of DON and NIV accumulation in barley grains without FHB symptoms. Therefore, the lowered accumulation of DON and NIV following late fungicide application may have been caused, at least in part, by preventing primary infection at the late stages. The continuous inoculum supply up to the time of harvest in both test years would support this possibility. Such late timing of fungicide application also may have reduced the amount of mycotoxin production in previously infected grain, considering the rather high efficacy in controlling DON + NIV (49.7 and 42.4% in 2005 and 52.0 and 54.7% in 2006 by application at 21 and 29 to 30 DAA, respectively). Even if this speculation is true, it remains unclear whether fungicide application at these late stages reduces the rate of mycotoxin production by interfering with trichothecene synthesis or prevents fungal development accompanying mycotoxin production. Investigations of the increase in mycotoxins and fungal biomass after *F. graminearum* infection would provide practical information to elucidate the manner in which mycotoxin is produced by the fungus. This would be useful in the development of strategies to control mycotoxin accumulation.

Although the effectiveness of late-stage fungicide application in controlling mycotoxins without controlling disease was indicated, such late application can lead to residues in the harvested grain. Thus, for commercial barley production, the use of some fungicides at late stages may be restricted. Nevertheless, our results demonstrate the potential of the late timing of treatment for controlling mycotoxin contamination in barley.

To assess the effects of the timing of fungicide application, we adopted the inoculation method using colonized maize kernels and sprinkler irrigation. It would be difficult to assess the effects of fungicide application timing with experiments using spray inoculation or under natural infection conditions because, with such methods, the timing of spray inoculation or natural precipitation would affect the re-

sults. The inoculation method used in this study, which provides periodic moist conditions and generates *F. graminearum* spores throughout the testing season, would be most appropriate for the purposes of this study.

The disease level (FHB incidence and severity) in the no-fungicide control plots was moderately severe in 2005 but slight in 2006. This could mainly be attributed to the difference in the amount of sprinkler irrigation (15 min each time in 2005 versus 6 min in 2006). Because disease and mycotoxin levels in 2005 were high, we reduced the length of water application in 2006, intending to lower the disease level in that year. This should have shortened the moist period of the plant and the environment in 2006, which may have led to relatively less favorable conditions for ascospore production and fungal infection of the spikes. In fact, the number of *F. graminearum* spores observed during the test season was, on the whole, less in 2006 than in 2005. The difference in the density of spread of inoculum kernels (48 and 43 ml/m² in 2005 and 2006, respectively), although small, also may have contributed to the difference in the levels of ascospore production and disease severity between the test years.

Although the disease level observed in the field and mycotoxin levels in grain were higher in 2005 than in 2006, the percentage of DK in harvested grain was higher in 2006 than in 2005. Discolored kernels in barley are caused not only by *Fusarium* spp. but also by several other pathogens (21,28); therefore, it is possible that there was a contribution from a pathogen other than *F. graminearum* to the occurrence of DK in 2006.

Spent anther extrusion in cleistogamous barley may not be recognized without careful observation because the spent anthers that are extruded at the tip of the floret are very small. Therefore, to target the beginning of spent anther extrusion for chemical control in cleistogamous barley, it would be more efficient to know the relative timing of spent anther extrusion during the early stages of barley development. The relative timing of spent anther extrusion and the heading stage or anthesis can differ with cultivar and environment, although anthesis in cleistogamous barley is more difficult to identify than spent anther extrusion. Low temperatures usually delay grain development and, consequently, the timing of spent anther extrusion after anthesis or heading. In Japan, our preliminary observations indicate that the spent anther extrusion of cleistogamous barley usually begins 14 to 18 days after head emergence (ZGS 52 to 53; one-quarter of the inflorescence emerged in >50% of spikes) and 10 to 15 days after anthesis (ZGS 64 to 65). Although anthesis usually occurs after heading in barley (5), in some barley-growing regions, it occurs

before heading (22,38). In such situations, the relative timing of spent anther extrusion and heading in cleistogamous barley would be quite different from those in Japan.

Spent anther extrusion has not been given adequate attention thus far and it is poorly documented worldwide. Not only the timing but also the degree of extrusion may differ among cultivars or environments. It would be helpful, not only in Japan but also in other barley-growing regions, to observe spent anther extrusion in cleistogamous barley cultivars in order to understand the cultivars' response to FHB and determine the timing of chemical control against FHB and mycotoxin contamination.

The type of fungicide may affect the results of application. We used Topsin M (thiophanate-methyl, a benzimidazole), which is a broad-spectrum systemic fungicide with both preventive and curative properties. Thiophanate-methyl is converted to methyl benzimidazole carbamate (carbendazim), and this compound interferes with nuclear division in sensitive fungi. It is likely that the systemic and/or curative properties of Topsin M are critical to effectively control mycotoxin accumulation in grain, especially when applied at later stages. Further studies are needed to evaluate the efficacy of other fungicides with different properties or different modes of action in applications at the beginning of spent anther extrusion and at later stages in cleistogamous barley.

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LITERATURE CITED

1. Anonymous. 2002. Discussion paper on deoxynivalenol. [Agenda Item 16(g), CX/FAC 02/29.] Codex Committee on Food Additives and Contaminants, 34th Session (Rotterdam, 11–15 March 2002). Codex Alimentarius Commission FAO/WHO, Rome, Italy.
2. Anonymous. 2007. Annual Inventory of Registered Pesticides and Their Use. Japan Plant Protection Association, Tokyo. (In Japanese)
3. Anonymous. 2007. Annual Statistics on Pests and Pesticides. Japan Plant Protection Association, Tokyo. (In Japanese)
4. Atanasoff, D. 1920. *Fusarium*-blight (scab) of wheat and other cereals. J. Agric. Res. 20:1-40.
5. Briggs, D. 1978. Barley. Chapman and Hall, London.
6. Campbell, K. A. G., and Lipps, P. E. 1998. Allocation of resources: sources of variation in *Fusarium* head blight screening nurseries. Phytopathology 88:1078-1086.
7. Choo, T. M., Martin, R. A., Ho, K. M., Shen, Q., Fedak, G., Savard, M., Voldeng, H., Falk, D. E., Etienne, M., and Sparry, E. 2004. *Fusarium* head blight and deoxynivalenol accumulation of barley in eastern Canada: cultivar response and correlation analysis. Plant Dis.

- 88:837-844.
8. Dill-Macky, R. 2003. Inoculation methods and evaluation of Fusarium head blight resistance in wheat. Pages 184-210 in: *Fusarium Head Blight of Wheat and Barley*. K. J. Leonard and W. R. Bushnell, eds. American Phytopathological Society, St. Paul, MN.
9. Feekes, W. 1941. De tarwe en haar milieu. Versl. Techn. Tarwe Comm. 12:523-888.
10. Havlova, P., Lancova, K., Vanova, M., Havel, J., and Hajslova, J. 2006. The effect of fungicidal treatment on selected quality parameters of barley and malt. *J. Agric. Food Chem.* 54:1353-1360.
11. Heta, H., and Hiura, U. 1963. Varietal differences in resistance to head blight, *Gibberella zeae* (Schw.) Petch. Studies on disease-resistance in barley. XIII. *Nogaku Kenkyu* 49:177-187. (In Japanese)
12. Horsley, R. D., Pederson, J. D., Schwarz, P. B., McKay, K., Hochhalter, M. R., and McMullen, M. P. 2006. Integrated use of tebuconazole and Fusarium head blight-resistant barley genotypes. *Agron. J.* 98:194-197.
13. Ichinoe, M., Hagiwara, H., and Kurata, H. 1984. Distribution of trichothecene-producing fungi in barley and wheat fields in Japan. Pages 190-198 in: *Toxigenic Fungi: Their Toxins and Health Hazard*. H. Kurata and Y. Ueno, eds. Elsevier, Tokyo.
14. Ichinoe, M., Kurata, H., Sugiura, Y., and Ueno, Y. 1983. Chemotaxonomy of *Gibberella zeae* with special reference to production of trichothecenes and zearalenone. *Appl. Environ. Microbiol.* 46:1364-1369.
15. Ios, R., Belhadj, A., Menez, M., and Faure, A. 2005. The effects of fungicides on *Fusarium* spp. and *Microdochium nivale* and their associated trichothecene mycotoxins in French naturally infected cereal grains. *Crop Prot.* 24:894-902.
16. Jones, R. K. 2000. Assessments of Fusarium head blight of wheat and barley in response to fungicide treatment. *Plant Dis.* 84:1021-1030.
17. Jordahl, J., Meyer, S., and McMullen, M. 2002. Multiple infection events and split timing of Folicur fungicide applications for control of FHB in hard red spring wheat, durum wheat, and spring barley, 2002. Page 91 in: *Proc. 2002 Natl. Fusarium Head Blight Forum*, Erlanger, KY.
18. Kim, H. S., Lee, T., Dawlatana, M., Yun, S. H., and Lee, Y. W. 2003. Polymorphism of trichothecene biosynthesis genes in deoxynivalenol- and nivalenol-producing *Fusarium graminearum* isolates. *Mycol. Res.* 107:190-197.
19. Koizumi, S., Kato, H., Yoshino, R., Komada, H., Ichinoe, M., Umehara, Y., and Hayashi, N. 1997. Pathogenicity and ecology of *Fusarium* species associated with wheat and barley scab in Japan. *Bull. Inst. Compr. Agric. Sci. Kinki Univ.* 5:31-45.
20. Lee, T., Han, Y. K., Kim, K. H., Yun, S. H., and Lee, Y. W. 2002. *Tri 13* and *Tri 7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. *Appl. Environ. Microbiol.* 68:2148-2154.
21. Mathre, D. E. 1982. Compendium of Barley Diseases. American Phytopathological Society, St. Paul, MN.
22. McCallum, B. D., and Tekauz, A. 2002. Influence of inoculation method and growth stage on Fusarium head blight in barley. *Can. J. Plant Pathol.* 24:77-80.
23. McCallum, B. D., Tekauz, A., and Gilbert, J. 2004. Reaction of a diverse collection of barley lines to Fusarium head blight. *Plant Dis.* 88:167-174.
24. McMullen, M., Halley, S., Pederson, J., Moos, J., and Jordahl, J. 2000. Effects of application parameters on control of Fusarium head blight with fungicides. Pages 94-97 in: *Proc. 2000 Natl. Fusarium Head Blight Forum*, Erlanger, KY.
25. McMullen, M., Jones, R., and Gallenberg, D. 1997. Scab of wheat and barley: a re-emerging disease of devastating impact. *Plant Dis.* 81:1340-1348.
26. Mesterhazy, Á. 2003. Control of Fusarium head blight of wheat by fungicides. Pages 363-380 in: *Fusarium Head Blight of Wheat and Barley*. K. J. Leonard and W. R. Bushnell, eds. American Phytopathological Society, St. Paul, MN.
27. Meyer, S., Jordahl, J., and McMullen, M. 2006. Uniform fungicide trial results on HRS wheat and barley, Fargo, ND 2006. Page 14 in: *Proc. 2006 Natl. Fusarium Head Blight Forum*, Durham, NC.
28. Miles, M. R., Wilcoxson, R. D., Rasmussen, D. C., Wiersma, J., and Warnes, D. 1987. Influence of genotype and environment on kernel discoloration of Midwestern malting barley. *Plant Dis.* 71:500-504.
29. Mirocha, C. J., Abbas, H. K., Windels, C. E., and Xie, W. 1989. Variation in deoxynivalenol, 15-deoxynivalenol, 3-acetyldeoxynivalenol and zearalenone production by *Fusarium graminearum* isolates. *Appl. Environ. Microbiol.* 55:1315-1316.
30. Nakajima, T., and Yoshida, M. 2007. Mycotoxin productivity and virulence of *Fusarium graminearum* species complex causing Fusarium head blight on wheat and barley in the western part of Japan. *Jpn. J. Phytopathol.* 73:106-111. (In Japanese with English abstract)
31. Neate, S. M., McKay, K. R., and Halley, S. A. 2003. Split application of fungicides for increased control of FHB and DON on barley. Page 104 in: *Proc. 2003 Natl. Fusarium Head Blight Forum*, Bloomington, MN.
32. Parry, D. W., Jenkinson, P., and McLeod, L. 1995. *Fusarium* ear blight (scab) in small-grain cereals: a review. *Plant Pathol.* 44:207-238.
33. Paul, P. A., Lipps, P. E., Hershman, D. E., McMullen, M. P., Draper, M. A., and Madden, L. V. 2007. A quantitative review of tebuconazole effect on Fusarium head blight and deoxynivalenol content in wheat. *Phytopathology* 97:211-220.
34. Placinta, C. M., D'Mello, J. B. F., and Macdonald, A. M. C. 1999. A review of world contamination of cereal grains and animal feeds with *Fusarium* mycotoxins. *Anim. Feed Sci. Technol.* 78:21-37.
35. Pugh, G. W., Johann, H., and Dickson, J. G. 1933. Factors affecting infection of wheat heads by *Gibberella saubinetii*. *J. Agric. Res.* 46:771-797.
36. Ryu, J., Ohtsubo, K., Izumiyama, N., Nakamura, K., Tanaka, T., Yamamura, H., and Ueno, Y. 1988. The acute and chronic toxicities of nivalenol in mice. *Fundam. Appl. Toxicol.* 11:38-47.
37. Seo, J. A., Kim, J. C., Lee, D. H., and Lee, Y. W. 1996. Variation in 8-keto-trichothecenes and zearalenone production by *Fusarium graminearum* isolates from corn and barley in Korea. *Mycopathologia* 134:31-37.
38. Steffenson, B. J. 2003. Fusarium head blight of barley: impact, epidemics, management, and strategies for identifying and utilizing genetic resistance. Pages 241-295 in: *Fusarium Head Blight of Wheat and Barley*. K. J. Leonard and W. R. Bushnell, eds. American Phytopathological Society, St. Paul, MN.
39. Sugiura, Y., Watanabe, Y., Tanaka, T., Yamamoto, S., and Ueno, Y. 1990. Occurrence of *Gibberella zeae* strains that produce both nivalenol and deoxynivalenol. *Appl. Environ. Microbiol.* 56:3047-3051.
40. Sutton, J. C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Can. J. Plant Pathol.* 4:195-209.
41. Suzuki, T., Kurisu, M., Hoshino, Y., Ichinoe, M., Nose, N., Tokumaru, Y., and Watanabe, A. 1980. Production of trichothecene mycotoxins of *Fusarium* species in wheat and barley harvested in Saitama Prefecture. *J. Food Hyg. Soc. Jpn.* 21:43-49. (In Japanese)
42. Takahashi, M., Shibutani, M., Sugita-Konishi, Y., Aihara, M., Inoue, K., Woo, G., Fujimoto, H., and Hirose, M. 2008. A 90-day subchronic toxicity study of nivalenol, a trichothecene mycotoxin, in F344 rats. *Food Chem. Toxicol.* 46:125-135.
43. Takeda, K., and Heta, H. 1989. Establishing the testing method and a search for the resistant varieties to Fusarium head blight in barley. *Jpn. J. Breed.* 39:203-216. (In Japanese with English summary)
44. Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U. S., Sugiura, Y., and Ueno, Y. 1988. Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. *J. Agric. Food Chem.* 36:979-983.
45. Tanaka, T., and Sugiura, Y. 2003. Levels and pattern of contamination with trichothecenes in cereal grains and their risk assessment. *Mycotoxins* 53:119-121.
46. Togawa, M. 1994. Selective medium for isolation of *Fusarium graminearum*. *Soil Microorg.* 44:77-88. (In Japanese with English abstract)
47. Ueno, Y., Aikawa, Y., Okumura, H., Sugiura, Y., Nakamura, K., Masuma, R., Tanaka, T., Young, C. J., and Savard, M. E. 1997. Trichothecenes produced by *Fusarium* species Fn 2B. *Mycotoxins* 45:25-31.
48. Vanova, M., Hajslova, J., Havlova, P., Matusinsky, P., Lancova, K., and Spitzerova, D. 2004. Effect of spring barley protection on the production of *Fusarium* spp. mycotoxins in grain and malt using fungicides in field trials. *Plant Soil Environ.* 50:447-455.
49. Yoshida, M., Kawada, N., and Nakajima, T. 2007. Effect of infection timing on Fusarium head blight and mycotoxin accumulation in open- and closed-flowering barley. *Phytopathology* 97:1054-1062.
50. Yoshida, M., Kawada, N., and Tohnooka, T. 2005. Effect of row type, flowering type and several other spike characters on resistance to Fusarium head blight in barley. *Euphytica* 141:217-227.
51. Yoshizawa, T. 1997. Geographic difference in trichothecene occurrence in Japanese wheat and barley. *Bull. Inst. Compr. Agric. Sci. Kinki Univ.* 5:23-30.
52. Yoshizawa, T., and Jin, T. Z. 1998. Trichothecene occurrence in Japanese wheat and barley—its characteristics. *Mycotoxins* 47:15-18.
53. Yoshizawa, T., Kohno, H., Ikeda, K., Shinoda, T., Yokohama, H., Morita, K., Kusada, O., and Kobayashi, Y. 2004. A practical method for measuring deoxynivalenol, nivalenol, and T-2 + HT-2 toxin in foods by an enzyme-linked immunosorbent assay using monoclonal antibodies. *Biosci. Biotechnol. Biochem.* 68:2076-2085.
54. Zadoks, J. C., Chang, T. T., and Konzak, C. F. 1974. A decimal code for the growth stages of cereals. *Weed Res.* 14:415-421.
55. Zhou, X., Chao, M., and Liang, X. 1991. Screening and testing of barley varieties for scab resistance. *Acta Phytophylacica Sin.* 18:261-265. (In Chinese with English summary)