Rainfastness of Prothioconazole + Tebuconazole for Fusarium Head Blight and Deoxynivalenol Management in Soft Red Winter Wheat

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Abstract

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Fungicides are most warranted for control of Fusarium head blight (FHB), a disease of wheat caused by the fungal pathogen *Fusarium graminearum*, when wet, rainy conditions occur during anthesis. However, it is unclear whether rainfall directly following application affects fungicide efficacy against FHB and its associated toxin, deoxynivalenol (DON). The objective of this study was to determine the rainfastness of the fungicide tebuconazole + prothioconazole and the residual life of tebuconazole when applied to wheat spikes at anthesis in combination with the nonionic surfactant Induce. Three field experiments were conducted during 2012 and 2013 in Wooster, OH. Simulated rainfall of a fixed intensity and duration was applied to separate plots at five different times after the fungicide treatment (0, 60, 105, 150, or 195 min). Spike samples were collected at 4-day intervals after fungicide application and assayed for tebuconazole residue. A similar set of greenhouse experiments was conducted using six post-fungicide-application rainfall timing treatments (0, 15, 30, 60, 120, or 180 min). All experiments were inoculated at anthesis with spores of *F. graminearum*, and FHB index (IND) and DON were quantified. In four of the five experiments, all fungicide-treated experimental units (EUs) had significantly lower mean IND and DON than the untreated check, regardless of rainfall treatment. Among rainfall treatments, EUs that received the earliest rains after fungicide application tended to have the highest numerical mean IND and DON, but were generally not significantly different from EUs that received later rain or fungicide without rain. In both years, fungicide residue on wheat spikes decreased rapidly with time after application, but the rate of reduction varied somewhat between years, with a half-life of 6 to 9 days. Rainfall treatment did not have a significant effect on the rate of residue reduction or the level of residue at a fixed sampling time after fungicide application. In this study, tebuconazole + prothioconazole mixed with a nonionic surfactant was fairly rainfast for a fixed set of rainfall characteristics, and tebuconazole residue did not persist very long after application on wheat spikes.

Fusarium head blight (FHB), an important disease of wheat and other small grain crops, is primarily caused by the fungal pathogen *Fusarium graminearum* (teleomorph: *Gibberella zeae*) in North America. The economic impacts of this disease have been devastating, with a reported 3 billon dollars in losses in the 1990s alone (32). Losses are largely due to reduced grain yield and seed quality and are exacerbated by contamination of grain with mycotoxins, particularly deoxynivalenol (DON). DON has shown toxicity to both humans and livestock. It inhibits protein synthesis and causes vomiting, feed refusal, and other acute symptoms (5,24). Because of health concerns, the U.S. Food and Drug Administration has set a 2 ppm DON threshold for wheat grain and 1 ppm for finished wheat products destined for human consumption (27). Consequently, grain with DON levels above 2 ppm may be rejected completely or priced down at grain elevators.

Much research effort has been dedicated to the management of FHB and DON with fungicides in an effort to minimize grain yield and quality losses. The triazole family of fungicides has been shown to be the most effective against FHB and DON (3,18,22), with the highest levels of efficacy achieved when applications are made after head emergence, around early anthesis (17). However, among the triazoles, considerable variation in efficacy has been observed from one active ingredient to another and among studies (22). An imperative need to better evaluate the overall efficacy of fungicides against FHB and DON led to the establishment of uniform fungicide trials (UFTs) through the U.S. Wheat and Barley Scab Initiative (USWBSI) (21). Based on a quantitative synthesis of results from more than 100 UFTs, researchers discerned that the greatest reduction in FHB index and DON when relying on a single anthesis application was obtained with tebuconazole + prothioconazole (Prosaro 421 SC; Bayer CropScience, Research Triangle Park, NC), metconazole (Caramba 90 SL; BASF Corporation Agricultural Products, Research Triangle Park, NC), or prothioconazole (Proline 480 SC; Bayer CropScience) (22).

Prosaro is now one of the industry standards for FHB and DON management, and results from a meta-analysis of data from USWBSI uniform integrated management trials showed that when used in combination with a moderately resistant cultivar, this fungicide contributed to reducing both FHB and DON by over 70% relative to the untreated, susceptible check (31). However, despite its efficacy and very clear indications that it has to become an integral part of any FHB/DON management program, commercial utilization of Prosaro following current application guidelines has been somewhat of a challenge. In particular, producers have not always been able to apply this fungicide at the recommended flowering growth stage. This is either because wet, rainy field conditions during anthesis make it difficult, if not impossible, to make ground applications or simply because it is not always easy to determine the exact flowering date. The issue of rainfall around the time of anthesis, conditions under which a fungicide is most warranted for FHB management, is also of concern to producers from the standpoint of its effect on efficacy, and could affect their decision to make an application.

What is the effect of rainfall on absorption, efficacy, and the persistence of residue of Prosaro when it is applied during, or directly before, a rainfall event? Rainfall may negatively affect the performance of a fungicide by washing it off of the plant surface or diluting it to a less effective concentration. The extent to which this occurs depends on several factors, including when and how much it rains after the product is applied, the formulation of the product,

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and whether it is applied with a surfactant, the physicochemical properties and biokinetic behavior of the fungicide, and physical characteristics of the plant part being treated (6,8,12,25,26,28). To date, no studies have been published evaluating the rainfastness of Prosaro when applied to wheat spikes under field conditions, and the existing product label provides no such information. The objectives of this study were to: (i) determine the rain-fast time (the time needed between an application and a rain event for the product to maintain its effectiveness) of tebuconazole + prothioconazole (Prosaro) when applied at anthesis, and (ii) evaluate the persistence of one of the active ingredients in Prosaro (tebuconazole) in wheat spikes over the weeks following application.

Materials and Methods

Establishment and design of the experiments. *Field experiments.* Three experiments were conducted during the 2011–12 and 2012–13 growing seasons at the Ohio Agricultural Research and Development Center in Wooster. In 2011, plots of Hopewell, an awnless, susceptible soft red winter wheat (SRWW) cultivar, were planted on 6 October. In 2012, two experiments were established at the same research station, one using Hopewell and the other using Pioneer 25R45, an awned, susceptible SRWW cultivar. All plots were planted on 25 September. In both years, plots were planted with a Kincaid planter (Great Plains research drill, Kincaid Equipment Manufacturing, Haven, KS) at a seeding rate of 4×10^6 seeds/ha. Each experimental unit (plot) was seven rows wide and 3 m long, with a spacing of 19 cm between rows. Plots were managed in terms of fertilizer application and pest control according to standard agronomic practices for the Wooster region (20).

Greenhouse experiment. Four controlled-environment studies, two spray-inoculated and two point inoculated (as described below), were performed to complement the field studies in an effort to better assess the effects of rainfall on the efficacy of Prosaro against FHB. The experiments were conducted in 2013, the first two from 10 March to 3 May and the second from 24 April to 1 July. Seeds of awnless, FHB-susceptible SRWW cultivar Cooper were sown on 12 December 2012 and again on 3 January 2013 in plastic trays with a row spacing of 4 cm. Seeds were allowed to germinate in the greenhouse for 2 weeks, treated with Osmocote 14-14-14 (Scotts Miracle-Grow, Columbus, OH), and then transferred to a cold room at 3°C for 10 weeks to vernalize. After vernalization, individual seedlings were transferred to cones (Stuewe and Sons, Inc., Corvallis, OR) and placed in the greenhouse (average daily temperature of 26°C) until they were ready for inoculation. At approximately Feekes growth stage 8 (flag leaf emergence), plants were thinned by clipping all but the primary tillers and staked using a piece of bamboo to support growth.

A randomized complete block design was used in both the field and greenhouse experiments, with four replicate blocks in the field and three in the greenhouse. For the greenhouse experiments, each experimental unit consisted of 10 plants, and flowering date served as the blocking factor.

Fungicide treatment, rainfall simulation, and inoculation. *Field experiments*. A single, uniform application of Prosaro 421 SC (at 100 g each of tebuconazole and prothioconazole per hectare) was made at anthesis (Feekes 10.5.1) to all but one plot in each block. A nonionic surfactant (Induce, Helena Chemical Company, Collierville, TN) was added to the fungicide at a rate of 0.125% vol/vol. In year one (2012), applications were made using a tractor-mounted sprayer equipped with three pairs of flat fan XR8001VS nozzles (TeeJet Technologies, Dillsburg, PA) spaced 50 cm apart and mounted at an angle of 45° from the horizontal. The system was calibrated to deliver the fungicide at a rate of 187 liters/ha at 200 kPa. In year two, similar spray parameters were used in terms of volume and pressure, but applications were made using a CO_2-pressurized backpack sprayer (R&D Sprayers, Opelousas, LA) with three TT110015-VP nozzles (TeeJet Technologies) equally spaced on a 100-cm-long boom. Plots that did not receive the fungicide application (referred to as Check1) were used as references for efficacy evaluation.

Following fungicide application, plots were subjected to simulated rainfall. In 2012, there were four simulated rainfall treatments—rainfall at 60, 105, 150, or 195 min after Prosaro application—plus a no-rainfall reference treatment (referred to as Check2). In 2013, an additional simulated rainfall treatment, imposed immediately after the fungicide was applied (0 min), was included in the two experiments. However, during this year the fungicide-treated, no-rainfall check was not included in the design. In the first year, rainfall was simulated with portable rain simulators constructed using risers, nozzles, valves, and PVC piping (based on simulators developed by Humphry et al. [7] and Dufault and Isard [2]). Each simulator was equipped with a Rain Bird nozzle (R13-18F, Rain Bird Corporation, Azusa, CA) powered by a CO_2 pressure system, regulated by valves, and calibrated to deliver rainfall at an intensity of approximately 39 mm h^{-1} at 207 kPa over a 3 × 1 m section of each plot. The simulated rainfall event lasted approximately 6 min, delivering an average of 3.9 mm of water during that period. In the second year, four rain simulators were custom built (Steinn-Way Equipment, Apple Creek, OH) from galvanized steel piping. Each simulator was 3 m long, 1.5 m wide, and 1.8 m high, and mounted on wheels. They were equipped with two quick fulljet nozzles (Spraying Systems Co., Glendale Heights, IL) spaced 1 m apart on a 1-m-long boom and powered by pressurized CO_2 set to 200 kPa. The system was calibrated to deliver approximately 6.15 mm of rainfall for 190 s, at an intensity of 116.5 mm h^{-1}. In all experiments, there was one simulator per block.

Plots were spray-inoculated with a spore suspension of *F. graminearum* on the evening of the day of fungicide application and rainfall treatments. Inoculum consisted of a mixture of equal proportions of ascospores and macroconidia from 10 Ohio isolates of *F. graminearum* (OHWAY1619, OHWAY627, OHWOO613, OHVAN4619, OHDEL3616, OHSHE6613, OHBUC6613, OHPAU2613, OHBUT611, and OHAUG621) of the 15-acetyldeoxynivalenol (15-ADON) chemotype (D. Schmale, *personal communication*). Ascospores were produced by transferring plugs from stock cultures onto carrot agar. After approximately 1 week of growth, visible mycelia were scraped from the plate with a rubber policeman and discarded, and plates were moved to a dark room to induce perithecial development. After 2 weeks, plates were flooded with sterile water and agitated to harvest ascospores. Macroconidia were obtained from isolates of *F. graminearum* plated onto mung bean agar (MBA). Mycelial plugs were taken from isolates grown on Komada selective medium and aseptically transferred to MBA. After approximately 10 days, 500 µl of sterile water was added to each plate, and spores were dislodged using a rubber policeman and harvested. A final concentration of 100,000 spores ml^{-1} was achieved for each spore type using a hemacytometer. A 1:1 mixture of ascospores and macroconidia plus 2.5% Tween 20 (Sigma-Aldrich, St. Louis, MO) was applied to each plot with a CO_2-pressurized backpack sprayer (R&D Sprayers) equipped with three flat fan TT110015-VP nozzles (TeeJet Technologies) spaced 50 cm apart on a 100-cm-long boom and calibrated to deliver 750 ml of inoculum per plot at 200 kPa. A set of untreated, inoculated plots were kept as references against which all treatments were compared.

Greenhouse experiments. A similar set of simulated rainfall treatments to those used in the field were used in the greenhouse; however, slightly shorter time intervals between fungicide application and rainfall treatments and between successive rainfall treatments were tested. Moreover, the greenhouse experiments allowed for the assessment of rainfastness under relatively dryer and more uniform conditions than those commonly encountered in a wheat field during early-morning applications. The treatments were: simulated rainfall applied at 0 (immediately), 15, 30, 60, 120, or 180 min after fungicide application. A single rainfall simulator was used to minimize simulator-to-simulator variability, and all the rainfall treatments were applied at the same time to plants previously treated with the fungicide. Prosaro applications were made with a hand-held sprayer when plants reached anthesis (Feekes

10.5.1) at a rate and volume comparable to the field applications. Plants in the different rainfall treatment groups were treated with the fungicide sequentially, beginning with the 180-min rainfall treatment, followed by the 120-, 60-, 30-, and 15-min treatments, and were all exposed to simulated rainfall at the same time as the 0-min treatment. For instance, if the fungicide was first applied to spikes in the 180-min rainfall treatments group at 0930 h, it was subsequently applied to those in the 120-, 60-, 30-, 15-min, and 0-min treatment EUs at 1030, 1130, 1200, 1215, and 1230 h, respectively; then all groups were subjected to simulated rainfall at approximately 1230 h.

Rainfall was simulated using a system similar to that described for the 2013 field experiments (a 1-m-long steel boom fitted with two quick fulljet nozzles spaced 1 m apart and powered by pressurized CO_2). The simulator was suspended from the roof of the greenhouse, with the nozzles positioned approximately 1.5 m above the spikes. The system was calibrated to deliver a uniform amount of rainfall over a 4-min period at 200 kPa. The average volume of water applied was 3.64 mm, giving an average simulated rainfall intensity of 54.59 mm h^{-1}. A set of plants that received the fungicide application but no rainfall treatment (Check2) and another set that received neither the fungicide nor rainfall treatments (Check1) were used as references against which the other rainfall × fungicide treatment combinations were compared.

Approximately 24 h after fungicide application, 20 plants were inoculated with *F. graminearum* macroconidia using two different inoculation methods (treated here as two separate experiments). One set of 10 plants (spikes) was point-inoculated and the other spray-inoculated. The spray inoculation experiments were used to evaluate the effect of simulated rainfall on the efficacy of Prosaro before infection, whereas the point-inoculated experiments served to evaluate the efficacy of the fungicide assuming that an infection had occurred, or at a minimum, that penetration had occurred. Essentially, the spray and point inoculation methods allowed us to evaluate the performance of the fungicide on the surface and within the spike, as influenced by rainfall treatment. The point inoculations were done using a suspension of 10^4 macroconidia ml^{-1}. The central spikelet of each inoculated spike was marked with a small dot in permanent marker, then the glume, lemma, and palea of a floret on the marked spikelet were gently drawn back and, using a pipette, 10 µl of the spore suspension was injected directly into the floret, with each plant receiving approximately 100 spores (4). For the spray inoculation method, a spore concentration of 5×10^4 spores ml^{-1} was applied to each spike using a hand-held sprayer held approximately 30 cm from the spike. A single spray was applied to one side of the spike, then it was rotated 180 degrees and given a second full spray. Each spike received approximately 3.6×10^3 spores using this method (estimating by converting the difference in weight between a sample of spikes before and after being sprayed to volume, and then multiplying by the spore concentration).

Data collection and analysis. *Fungicide residue analysis*. To determine the effect of the imposed rainfall treatments on fungicide residue over time under field conditions, samples of 20 spikes were collected from each plot at 4-day intervals, beginning on the day rainfall and fungicide treatments were applied and ending 3 weeks later. Samples were stored at –40°C until processed and analyzed for fungicide residue using gas chromatography-mass spectrometry (GC-MS). Clean wheat heads were spiked with known volumes of a 32.2 µg ml^{-1} laboratory standard solution of tebuconazole (Chem Service, West Chester, PA), which is one of the two active ingredients in Prosaro. The spiked wheat heads were then used to create a calibration curve. The concentrations of the standards ranged from 0.8 to 20.0 µg ml^{-1}, with the limit of quantitation for tebuconazole being 0.8 µg ml^{-1} on the instrumentation used.

Quantification and analysis of matrix matched tebuconazole standards and samples were performed on a Varian 3800 GC with a Saturn 2200 ion trap mass spectrometer. A fused silica Agilent (Santa Clara, CA) J & W Capillary DB-MS column (30 m × 0.25 mm ID × 0.25 µm film thickness) was employed. Helium (99.99% purity) was used as the carrier gas at a flow rate of 1.0 ml/min. The GC oven temperature was programmed to run from 60°C (1 min) to 290°C (3 min) at 10°C min^{-1}. The 1177 injector was used and maintained at 250°C with a 20% split. The transfer line was heated to 280°C. Prothioconazole, the other active component of Prosaro, was not analyzed in this study. Due to its chemical structure and relatively high vaporization point, this analyte is best quantified using liquid chromatography. Since only GC-MS was available at the time of this study, tebuconazole was the only active ingredient in Prosaro that was analyzed.

For residue extraction, each sample of 20 wheat spikes was divided into five subsamples of four spikes, and each subsample was placed into a preweighed 50-ml conical centrifuge tube (Fisher Scientific, Waltham, MA). Spikes were weighed, mechanically ground in approximately 20 ml of acetone (Fisher Scientific PowerGen Model 500 Homogenizer), and shaken for 2 h on an orbital shaker (Big Bill digital oscillator, Thermolyne Corporation, Dubuque, IA). Samples were then filtered using a 47-mm SPE manifold (Konts, Fisher Scientific). Wheat residue was discarded and the filtrate was evaporated, which concentrated the sample, allowing for easier detection. Samples were then diluted in distilled water, and solid-phase extraction was performed using Strata-X columns (Strata X 33u Polymeric Reversed Phase 100 mg/6 ml, Phenomenex, Torrance, CA) in a 24-port SPE vacuum manifold (Fisher Scientific). Elution of tebuconazole from the sorbent bed of the columns into preweighed collection tubes was performed using acetonitrile. Elutions were then weighed, transferred to GC vials, and analyzed on the GC-MS instrument. Mass of tebuconazole per four wheat spikes was obtained (µg g^{-1}).

Disease assessment and DON analysis. At approximately 3 weeks after inoculation (Feekes 11.2), FHB incidence (mean proportion of diseased spikes per sample) and index (mean proportion of diseased spikelets per spike) were rated on 80 to 100 spikes from each plot in the field experiments. In the greenhouse, all 10 spikes in each experimental unit were rated. Field plots were harvested with a plot combine (ALMACO SPC 20), and Fusarium-damaged kernels (FDK; the percentage of small, shriveled, and discolored kernels in a sample) were rated visually. Spikes in the greenhouse were hand-harvested and threshed using a table-top thresher. Samples of grain from each experimental unit, for both field and greenhouse experiments, were ground (Laboratory Mill 3033, Perten Instruments, Springfield, IL) and sent to the USWBSI-funded mycotoxin testing laboratory at the University of Minnesota for DON analysis by GC-MS.

Data analysis. Linear mixed model ANOVA (14) were performed to determine the effect of fungicide treatment on FHB incidence (INC), index (IND), FDK, and DON accumulation (ppm) as influenced by simulated rainfall. For the purpose of this analysis, each unique fungicide × rainfall treatment combination was considered a separate treatment (hereafter referred to as TREATMENT). In addition, repeated measures analysis was used to determine the effect of time on fungicide residue on wheat spikes, as influenced by imposed rainfall treatment. Disease intensity data (IND, INC, and FDK from the field experiment and IND from the greenhouse experiment) were arcsine-square-root-transformed (arcINC, arcIND, and arcFDK), and DON was log-transformed (logDON+1) prior to analysis to stabilize variances. Each field experiment was analyzed separately, whereas for the greenhouse experiments, the two spray-inoculation experiments were pooled together as were the two point-inoculation experiments. There was a separate analysis for spray- and point-inoculation experiments. Repetition of the experiment was considered a blocking factor. In all cases, TREATMENT was the fixed effect with block being the random effect. Linear mixed models were fitted using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC). The *lines* option in the *lsmeans* statement in GLIMMIX was used to compare least-squares means at the 5% level of significance.

Models for the effects of TREATMENT and sampling date on fungicide residue were also fitted using PROC GLIMMIX of SAS. Analyses were performed using log-transformed fungicide residue

level (logRES) as the dependent variable, TREATMENT (the between-subject treatment factor) and sampling date (the repeated or within-subject treatment factor) as fixed effects, and block as a random effect. Since repeated measures on the same experimental units are often correlated (14), the random *_residual_* statement and *type* option in GLIMMIX were used to account for, and model, the covariance structure of the within-subject data. After a series of model assessments with different covariance models, the first-order autoregressive, AR(1), model was selected as being appropriate for the characteristics of the fungicide residue data. This model is adequate when correlations decrease as observations become further apart in time. *Lsmeans* and contrast statements were used to compare means among treatments and sampling dates, and among treatments within sampling dates.

Since preliminary analyses (based on orthogonal polynomial contrasts and graphical exploration of the raw data) suggested that there was a negative linear relationship between residue (on a log scale) and sampling time, models were refitted with time as a continuous covariate to estimate regression parameters for the relationship between logRES and time. Data from the 2012 and 2013 experiments were modeled separately.

Results

Field conditions, Fusarium head blight, and deoxynivalenol. In 2012, all plots reached approximately 50% anthesis (Feekes 10.5.1) on 17 May when Prosaro was applied in the early morning and then inoculation in the evening. Average temperature and relative humidity (RH) during the time of fungicide and rainfall treatment application (0700 to 1100 h) were 10°C and 72%, respectively. In 2013, plots in experiment 1 (cv. Hopewell) reached anthesis on 24 May and in experiment 2 on 25 May. During the morning of 24 May when treatments were applied, average temperature was 6°C, with average RH of 75%. Average temperature and RH on the morning of 25 May were 9°C and 70%, respectively.

Arithmetic means for FHB index (IND), incidence (INC), FDK, and deoxynivalenol (DON) are summarized in Figure 1. Disease

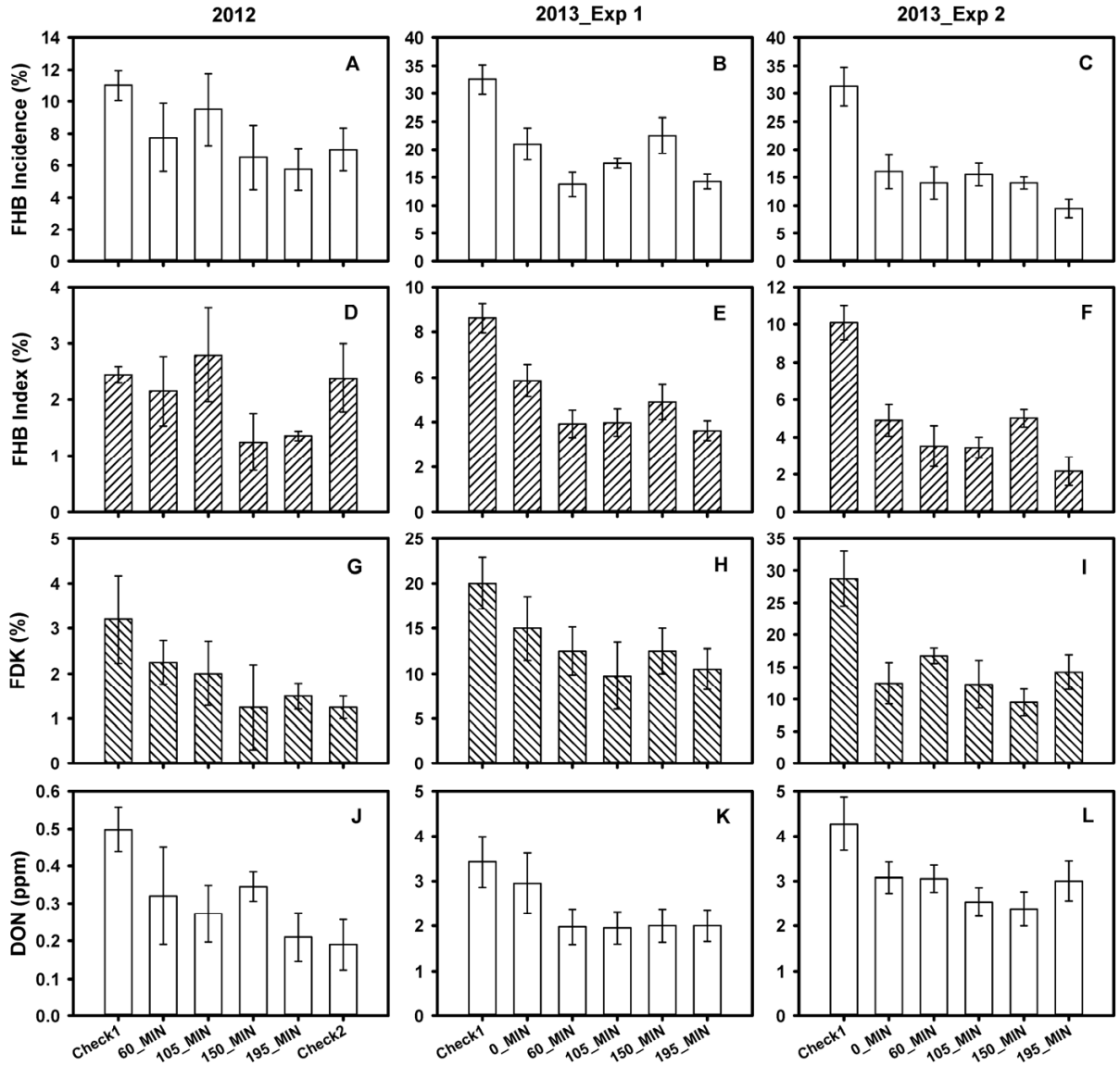

Fig. 1. Arithmetic mean Fusarium head blight (FHB) incidence **(A, B, and C),** mean percentage of diseased spike per sample of 80 to 100 spikes; index **(D, E, and F),** mean percentage of diseased spikelets per spike; Fusarium damaged kernels (FDK) **(G, H, and I),** mean percentage of small, shriveled, discolored kernels in a grain sample; and deoxynivalenol **(J, K, and L)** content of harvested grain (ppm) from all plots of soft red winter wheat exposed to simulated rainfall treatments at different times following the application of Prosaro 421 SC (at 100 g of each a.i./ha). Fungicide was applied at anthesis mixed with the nonionic surfactant Induce (0.125 vol/vol). Simulated rainfall treatments were applied at 0, 60, 105, 150, or 195 min after fungicide application. Check1 = no fungicide and no rainfall, and Check2 = fungicide application without simulated rainfall. Each rainfall event lasted approximately 6 min in 2012 and 3 min 10 s in 2013, at an intensity of 39 mm/h in the first year and 116.5 mm/h in the second. All plots were spray-inoculated at anthesis with a spore suspension (100,000 spores ml^{-1}) of *Fusarium graminearum* on the evening of the day of fungicide and rainfall treatment application. Each bar represents the treatment arithmetic mean from four replicate plots, and error bars are standard error of the mean.

and toxin levels were higher in 2013 than in 2012, with mean IND, INC, FDK, and DON ranging from 1.3 to 2.8%, 7.0 to 11.0%, 1.3 to 7.6%, and 0.19 to 0.35 ppm, respectively, in the first year, compared to 2.2 to 10.0%, 9.5 to 31.3%, 9.5 to 28.8%, and 1.95 to 4.28 ppm, respectively, in the second. In all three experiments, Check1, which received no fungicide and no rainfall treatment, had the highest mean disease and toxin levels. Averaged across experiments in 2013, mean IND, INC, FDK, and DON in Check1 were 9.4, 31.9, and 13.8%, and 3.85 ppm (Fig. 1). The corresponding means in 2012 were 2.5, 11.0, and 7.6%, and 0.50 ppm. In all experiments, there was generally a trend toward decreasing disease levels from the earliest to the latest rainfall treatment relative to the time the fungicide was applied. For instance, mean IND in the 0_MIN treatment (rain immediately after fungicide application) for experiments 1 and 2 in 2013 were 5.9 and 4.9%, compared to 3.6 and 2.2% in the 195_MIN treatment (rain 195 min after fungicide application).

Based on results from linear mixed model analysis of variance, in 2012, the year with the lowest levels of disease and toxin, the effect of TREATMENT was not statistically significant for any of the measured responses (Table 1). There was, however, a statistically significant TREATMENT effect on arcINC and arcIND in both experiments in 2013 (Exp1 and Exp2) (P = 0.001), and on arcFDK in Exp2, but not on DON in either of the experiments (Table 1). In both Exp1 and Exp2, mean IND and INC (on the arcsine-transformed scale) were significantly lower in all fungicide-treated plots than in plots that did not received the fungicide treatment (Check1, Table 2). Among the rainfall treatments, 0_MIN, the treatment that received rain immediately after fungicide application, had significantly higher mean arcIND than all but one of the later rainfall treatments (150_MIN) in Exp1. In Exp2, mean arcIND for 0_MIN was numerically higher than the other rainfall treatments (other than 150_MIN), but the difference was only statistically significant for the 195_MIN treatment (rain 195 min after fungicide application). Similar trends were observed for arcINC and arcFDK, with the 0_MIN rainfall treatment having numerically, but not always statistically, higher means than the later rainfall treatments.

Greenhouse conditions, Fusarium head blight, and deoxynivalenol. Average temperature during the time of treatment application, which spanned the 3 h required to make all applications, was 32.3°C. Mean IND in the experimental unit that received neither fungicide nor rainfall treatments (Check1) was 57% in the spray-inoculation experiments and 18% in the point-inoculation experiment (Fig. 2). The corresponding mean IND for fungicide-treated experimental units ranged from 5.3 to 9.6% and from 3.9 to 11.0% for the spray- and point-inoculation experiments, respectively. For DON, means for Check1 were 14.4 and 3.12 ppm for the spray- and point-inoculated experiments, respectively. Means for fungicide-treated experimental units ranged from 0.05 to 0.16 ppm for the spray-inoculated and 0.20 to 0.57 ppm for point-inoculated experiments.

There was a statistically significant effect of TREATMENT on IND and DON for both the spray and point inoculation experiments (Table 1), based on results from linear mixed model ANOVA. Means and multiple comparison contrasts are detailed in Figure 2. All treatments that received a fungicide application, regardless of whether and when they were subjected to simulated rain following that application, had significantly lower mean IND (on the arcsine-transformed scale) and DON (on the log-transformed scale) than the no-rain, no-fungicide check (Check1). For the spray inoculation experiment, no rainfall treatment was significantly different from Check2, the treatment that received a fungicide application without being subjected to simulated rain for either arcIND or logDON. However, for the point-inoculation experiment, the 0_MIN rainfall treatment had significantly higher mean IND that Check2.

Table 1. Probability values (significance level) from linear mixed model analyses of the effect of rainfall treatments on Fusarium head blight index, incidence, Fusarium damaged kernels, and deoxynivalenol (ppm) control with tebuconazole + prothiconazole fungicide (Prosaro 421 SC at 100 g of each a.i./ha), for each year of field experiments, as well as greenhouse experiments conducted in Wooster, OH

	Field[w]			Greenhouse[x]	
Response[y]	2012	2013 Exp1	2013 Exp2	Point	Spray
Index	0.264	0.001	<0.001	<0.001	<0.001
Incidence	0.237	<0.001	0.001	…[z]	…
Fusarium damaged kernel	0.327	0.294	0.016	…	…
Deoxynivalenol	0.093	0.158	0.138	<0.001	<0.001

[w] Susceptible awnless cultivar Hopewell was used in 2012 and in experiment 1 in 2013 (Exp1), whereas susceptible awned cultivar Pioneer 25R45 was used in experiment 2 in 2013 (Exp2).

[x] Spray = spikes were spray-inoculated with a *Fusarium graminearum* macroconidia suspension containing 50,000 spores ml^{-1}, Point = spikes were point-inoculated by pipetting 10 µl of a macroconidia suspension containing 10,000 spores ml^{-1} into the central spikelet of each spike.

[y] Fusarium head blight index = mean proportion of diseased spikelets per spike, Fusarium head blight incidence = mean proportion of diseased spike per sample (80 to 100 spikes), Fusarium damaged kernels = mean proportion of small, shriveled, discolored kernels in a grain sample, and deoxynivalenol content of harvested grain (ppm).

[z] Response not quantified in the greenhouse experiments.

Table 2. Mean Fusarium head blight (FHB) index and incidence for field experiments conducted in 2013 in Wooster, OH to evaluate the effect of tebuconazole + prothioconazole fungicide (Prosaro 421 SC at 100 g of each a.i./ha) on FHB as influenced by rainfall treatment

	Index[w]		Incidence[w]	
Treatment[x]	2013 Exp1[y]	2013 Exp2[y]	2013 Exp1[y]	2013 Exp 2[y]
0_MIN	5.86 B	4.90 B[z]	21.00 B	16.00 B
60_MIN	3.93 C	3.53 BC	13.75 C	14.00 B
105_MIN	3.98 C	3.45 BC	17.50 BC	15.50 B
150_MIN	4.90 BC	5.01 B	22.50 B	14.00 B
195_MIN	3.63 C	2.16 C	14.25 C	9.50 B
Check	8.62 A	10.09 A	32.50 A	31.25 A

[w] Fusarium head blight index = mean percentage of diseased spikelets per spike, Fusarium head blight incidence = mean percentage of diseased spike per sample (80 to 100 spikes).

[x] Represents time after fungicide application that rainfall treatment was applied. Check = no rainfall and no fungicide. Each rainfall event lasted approximately 3 min 10 s, at an intensity of 116.5 mm/h.

[y] Susceptible awnless cultivar Hopewell was used in experiment 1 (Exp1), while susceptible awned cultivar Pioneer 25R45 was used in experiment 2 (Exp2).

[z] Means with the same letter are not statistically different (significance level $P \leq 0.05$). Statistical analyses and mean separation were based on arcsine-square-root-transformed index and incidence; however, for presentation, original raw data means are shown.

Fungicide residue. Residual tebuconazole levels (μg g^{-1}) on wheat spikes for each rainfall treatment over time are summarized in Figure 3. In each year, spikes were sampled at six times after initial application, 0, 4, 8, 12, 16, and 20 days. For all treatments, in both years, there was a rapid decrease in tebuconazole residue over the 3-week sampling period. In 2012, averaged across treatments, mean residue level on the day of treatment application was 2.79 μg g^{-1} (ranged from 2.46 to 3.11 μg g^{-1}), rapidly decreasing to 1.07 μg g^{-1} 8 days later and 0.34 μg g^{-1} by the end of the sampling period (20 days after fungicide application). Initial residues levels were lower in 2013 than in 2012, ranging from 0.99 to 1.74 μg g^{-1} at time zero, and decreased more gradually during the first 8 days after fungicide application in the latter than in the former year. At 20 days after the fungicide treatment, there was an average of 88% reduction in residue in 2012 compared to 75% in 2013.

Based on the linear mixed model repeated measures analyses, in both 2012 and 2013, the effects of TREATMENT and the interaction between TREATMENT and sampling time were not statistically significant ($P \geq 0.05$). The effect of sampling time on logRES was significant, however ($P < 0.001$) in both years. Pairwise comparisons of residue (logRES) amount at sample times were determined using *estimate* statements in the GLIMMIX procedure of SAS. In 2012, mean logRES at all later sampling time points (4, 8, 12, and 16 days after) was significantly lower than at time 0 (samples collected on the same day as the fungicide treatment), with *P* values ranging from 0.001 to <0.001. This is consistent with a pattern of exponential decay (Fig. 3A). In 2013, a similar trend was observed, albeit with a relatively slower rate of residue reduction at early sampling dates (Fig. 3B).

Because the interaction effect of sampling time and rainfall treatment was not statistically significant, the relationship between logRES and sampling time (TIME) was modeled in each year using the pooled data across all treatments as follows:

$$logRES = a + b \cdot TIME$$

where a = intercept (logRES at time zero) and b = slope (rate of logRES decrease per unit increase in time). The slope and intercept were –0.112 (se = 0.004) and 0.998 (se = 0.081), respectively, in 2012, and –0.078 (se = 0.003) and 0.412 (se = 0.035) in 2013. Figure 3C and D represents back-transformed predicted logRES over time in 2012 and 2013, showing an exponential decrease in tebuconazole residue on wheat spike in both years. The half-life of tebuconazole on the spike (time taken for residue to decrease by 50%) was estimated as log(0.5)/*b*. The half-life was approximately 6 days in 2012 and 9 days in 2013.

Discussion

To our knowledge, this was the first study to formally evaluate the rainfastness of the fungicide tebuconazole + prothioconazole (Prosaro) and persistence of tebuconazole in soft red winter wheat. This investigation was conducted in direct response to stakeholders' questions, since the product label provides no such information. According to information from other sources (web searches), this fungicide is rainfast "as soon as it dries", or any-

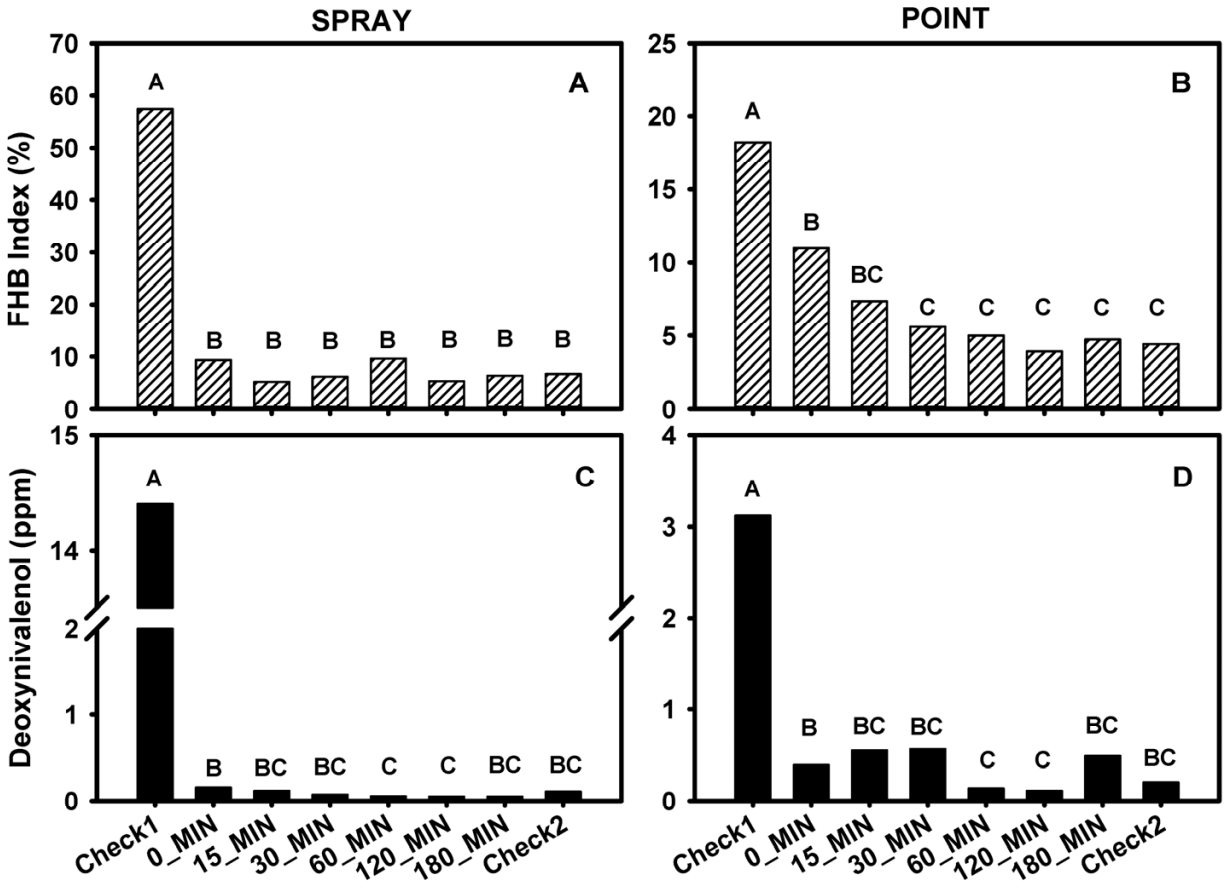

Fig. 2. Arithmetic mean Fusarium head blight index **(A and B)** and deoxynivalenol **(C and D)** for experimental units exposed to simulated rainfall treatments at different times after the application of Prosaro 421 SC (at 100 g of each a.i./ha) in two types of greenhouse experiments. Simulated rainfall treatments were applied at 0, 15, 30, 60, 120, or 180 min after fungicide application and the rainfall event lasted approximately 4 min, at an intensity of 54.59 mm/h. Check1 = plants were not treated with fungicide and received no rainfall, and Check2 = plants were treated with fungicide, but no rainfall. Spray (A and C) = spikes were spray-inoculated with a *Fusarium graminearum* macroconidia suspension containing 50,000 spores ml^{-1} and Point (B and D) = spikes were point-inoculated by pipetting 10 μl of a macroconidia suspension containing 10,000 spores ml^{-1} into the central spikelet of each spike. Means with the same letter are not statistically different at $P \leq 0.05$. Index was calculated as the mean proportion of spikelets per spike displaying visual symptoms (n = 10, per replicate). Statistical analyses and mean separation were based on arcsine-square-root-transformed index and log-transformed deoxynivalenol data, however, for presentation, original raw data means are shown. Each bar represents treatment mean of six replicates (two blocks [experiments or repetitions] and three replications per experiment).

where from 15 min to 2 h after application. Unfortunately, there appears to be no adequate published data to support any of these claims. It is well known that FHB epidemics are often severe when rainfall occurs around anthesis (Feekes 10.5.1) (15,16), since spikes are most susceptible to *F. graminearum* at this growth stage. Therefore, a single application of a fungicide is recommended at this time to manage this disease and toxins associated with it (10,17). So, from a practical management standpoint, it is critical for producers to understand what the effects of rainfall shortly after application are on efficacy against FHB and DON. Given the time of the day when fungicides are usually applied, the field conditions under which applications are most warranted, the moderate efficacy of this fungicide against FHB and DON (22,31), and the price paid for reduced grain quality due to FHB and DON (16,32), it is understandable if producers question the value of using a fungicide during rainy periods to manage this disease-toxin complex.

The rainfast results presented herein address several key questions pertaining to rainfall effect on the performance of Prosaro, when used according to standard recommendations (applied at 50% anthesis, at 475 ml/ha, and using a nonionic surfactant). The first relates to the timing of simulated rainfall events relative to fungicide application, which is somewhat different from rainfast-related studies in other systems that focus mainly on the amount and duration of rainfall on rainfastness (8,28). The second question addressed here relates to rainfastness under relatively dry and uniform conditions, such as those typical of a greenhouse, as opposed to wetter and variable conditions, such as in the wheat field during early-morning applications. A third question pertains to rainfastness in relation to infection by the pathogen.

Results from the field experiments conducted in 2013 showed that although the canopy was wet from dew at the time of fungicide and rainfall treatment applications (based on in-field data collected using surface wetness sensors), all fungicide-treated plots had significantly lower mean levels of IND than the untreated check, regardless of the rainfall treatment. Among the plots subjected to simulated rainfall treatments, only those that received rain immediately after fungicide application (0_MIN) tended to have significantly higher mean IND than the plots that received the latest rainfall application (195_MIN). This suggests that there may have been some 'wash off' before penetration for the 0_MIN treatment, but it probably was not sufficient to reduce efficacy relative to most of the other treatments that received later rainfall application. By 60 min (60_MIN) after fungicide application (the next rainfall treatment time point), the fungicide may have had sufficient time to become absorbed by the plant, causing this treatment to show a similar level of efficacy to the 195_MIN treatment. In fact, the efficacy of Prosaro (based on percent control) against IND and DON relative to Check1 (no-fungicide, no-rainfall) in plots subjected to simulated rainfall between 60 and 195 min after application was comparable to results from previous studies in which this same fungicide was applied in the absence of rainfall at anthesis (21,22), with mean percent control of 58 and 40% for IND and DON, respectively.

The overall efficacy of Prosaro (magnitude of the fungicide effect) under relatively dry and uniform greenhouse conditions was greater than under wetter, more variable field conditions. Averaged across comparable rainfall treatments (rainfall between 60 and 195 min after fungicide application), percent IND and DON control

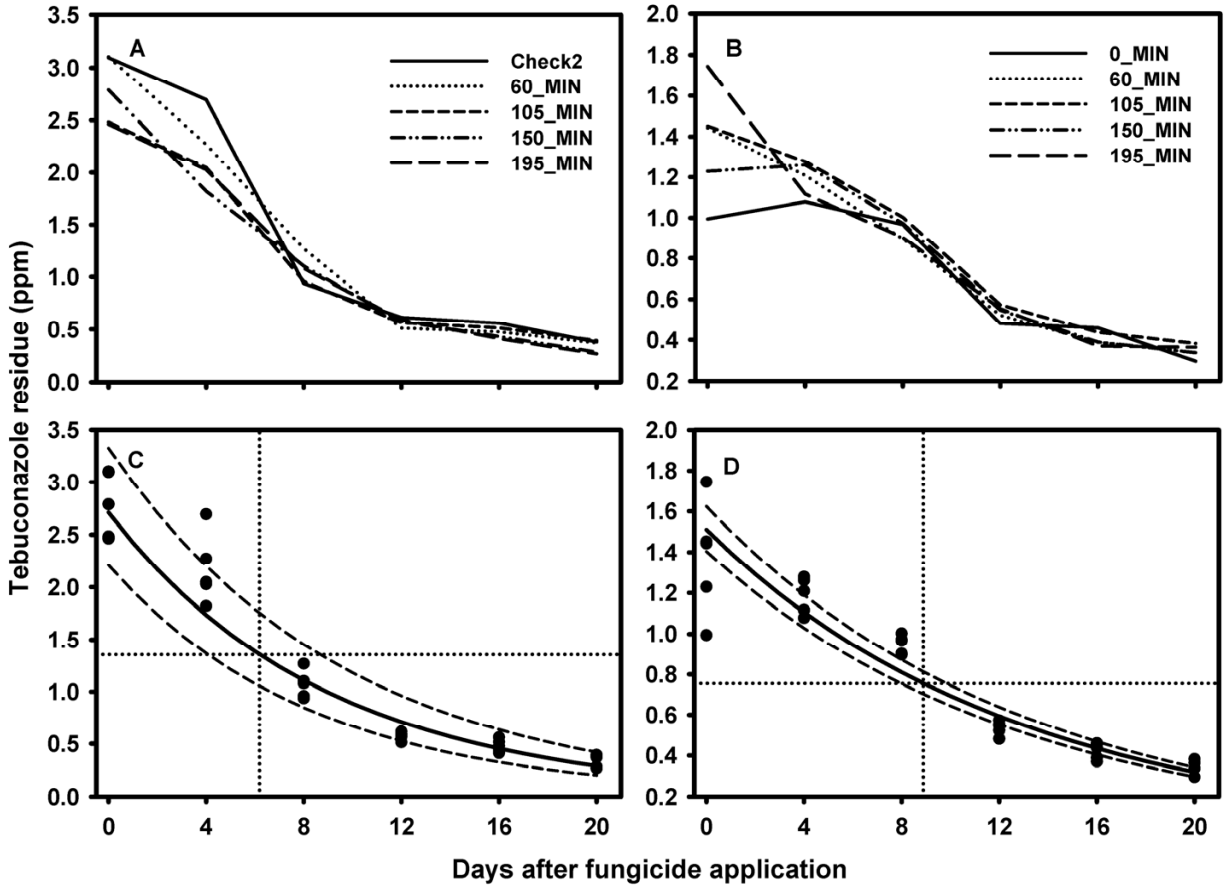

Fig. 3. Temporal change in residue of tebuconazole fungicide active ingredient on wheat spikes from plots exposed to simulated rainfall treatments deployed at different times after the fungicide was applied in field experiment conducted in 2012 **(A and C)** and 2013 **(B and D)**. Rainfall treatments were applied at 0 (in 2013 only), 60, 105, 150, or 195 min after fungicide application and each rainfall event lasted approximately 6 min in 2012 and 3 min 10 s in 2013, at an intensity of 39 mm/h in the first year and 116.5 mm/h in the second. Check2 = plots treated with fungicide without being exposed to simulated rain. A and B are plots of the raw data, whereas C and D show back-transformed predicted residue level (solid line) and the upper and lower limits of the 95% confidence interval (broken lines) from linear mixed model regression analysis of the relationship between log-transformed residue and time. Each dot in C and D represents the mean of 20 wheat spikes sampled from each plot. The reference lines show the half-life; the time (vertical reference line) required for tebuconazole residue on the spike to decrease by 50% (horizontal reference line).

relative to the non-fungicide-treated control (Check1) was much greater in the greenhouse than in the field. This could be due to several factors, including greater fungicide wash-off from a wetter canopy in the field that a drier canopy in the greenhouse, especially since the simulated rainfall amount and intensity were much greater in the field (6.16 mm at 116.6 mm/h) than in the greenhouse (3.64 mm at 54.59 mm/h). In addition, since experimental units in the greenhouse consisted of cohorts of 10 spikes at the same growth stage, compared to hundreds of spikes at slightly different growth stages in field plots, Prosaro was probably better able to protect spikes at the critical anthesis growth stage in the greenhouse than in the field, where spikes on secondary tillers may have been treated before anthesis when fungicides are generally less effective against FHB and DON (29,34). However, in terms of rainfastness (the time needed between an application and a rain event for the product to maintain its effectiveness relative to Check1), somewhat similar trends to those observed in the 2013 field experiments were observed in the greenhouse. All fungicide-treated experimental units (EUs), regardless of inoculation method and timing of rainfall events, had significantly lower levels of IND than Check1. Mean DON was also numerically lower in fungicide-treated EUs than in the untreated check both in the field and the greenhouse, but the difference was only statistically significant in the greenhouse experiments.

Point- and spray-inoculated greenhouse experiments provided information pertaining to the effect of shorter time intervals between fungicide application and rainfall treatment on rainfastness, as well as rainfastness in association with the infection process. All EUs, with the exception of those that received rain immediately after fungicide application (0_MIN) in the point-inoculated experiment, had comparable levels of IND to EUs that received the fungicide application without being subjected to simulated rain (Check2). For the point-inoculated experiment alone, fungicide-treated EUs subjected to rain between 0 and 15 min after application had 56% lower IND than Check1, compared to Check2 which had 76% lower IND than Check1, and EUs exposed to rain between 60 and 180 min after application with 75% lower IND than Check1. This could be attributed to the fact that rainfall immediately after fungicide application likely resulted in wash-off and reduced absorption that were sufficient to decrease efficacy relative to EUs exposed to rainfall after 30 min or EUs treated with Prosaro without being subjected to simulated rain. This difference between the point- and spray-inoculated experiments could be interpreted to mean that rainfall within the first 15 min after Prosaro application likely has a greater potential to affect the curative rather than the protective mode of action of this fungicide. Absorption is important for targeting the pathogen beyond the outer layers of the spike (the curative effect [9,19]) as was mimicked by the point-inoculation process.

The retention of Prosaro residue on or in wheat spikes after application has direct relevance for the control of secondary *F. graminearum* infection, whether this is due to late infection of spikes on primary tillers or to infection of spikes on late-developing secondary tillers. Although FHB is generally considered a monocyclic disease, secondary or late infections do occur, affecting DON accumulation (1,13,23,33). In the U.S. wheat production system, it is usually not economical to make multiple fungicide applications (22) in order to control any possible secondary infections; therefore it is important to evaluate post-treatment residue levels to determine whether a single anthesis application would be sufficient to control late *F. graminearum* infections (as well as other late-season diseases). Residue persistence, however, is often missing from fungicide labels. Anecdotal reports (based on web searches) suggest a residual life of 7 to 21 days for Prosaro, but again, there are no published data to support such claims. In addition, since the residual life of a fungicide (and pesticides in general) is influenced by weather, especially rainfall (30), we wanted to determine whether the temporal change in tebuconazole residue on wheat spikes was influenced by the imposed rainfall treatments. Our results showed that, on average, residue levels decreased by 50% within 6 to 9 days (the half-life) after fungicide application, regardless of rainfall treatment. Further research is needed to determine whether this is sufficient to reduce efficacy if infection occurs late (6 or more days after fungicide application). Moreover, the fact that the majority of the grain yield- and quality-impacting natural *F. graminearum* infections usually occur during a narrow 5- to 6-day window close to anthesis (the period of greatest host susceptibility), it is unclear whether 50% of the applied fungicide dosage will be enough to provide adequate protection of the spikes during anthesis.

Based on our findings, it seems reasonable to conclude that tebuconazole + prothioconazole (Prosaro 421 SC at 100 g of each a.i./ha) is fairly rainfast when applied with the surfactant Induce (0.125% vol/vol), with no substantial decrease in efficacy when a rainfall event occurs more than 15 min after fungicide application under dry and relatively uniform greenhouse conditions and 60 min under wetter and more variable field conditions. However, the conclusions drawn from this study are specific to the application parameters used, as well as the environmental conditions under which the experiments were conducted. Our focus here was the timing of rainfall events of fixed intensity and duration, but as other studies have shown for other systems, rainfastness may vary with the amount and duration of rainfall (12,28). However, since we used high rainfall intensities (39 to 116.6 mm/h) in the current investigation, we anticipate that our results will hold for a wide range of rainfall of similar duration. The use of the spray adjuvant may have contributed to the rainfastness of Prosaro observed here, as the purpose of a surfactant is to increase adhesion of the fungicide to the target tissue and subsequently increase efficacy (11,26). Because of the applied nature of this study, we only applied fungicide with surfactant because it was important to mimic actual field applications as closely as possible. Results may vary if Prosaro is applied without a surfactant or with a different surfactant, as have been observed in other rainfast studies (6,25). In addition, it would be of interest to know whether the observed rainfastness was due to one or both actives in Prosaro. Moreover, further research is also needed to determine whether the temporal change in the residue of prothioconazole, the other active ingredient in Prosaro, will be different from that observed for tebuconazole. Even though they are both DMIs with a similar mode of action, it is possible that rainfastness and persistence (residue half-life), which are both functions of the rate of absorption and translocation (among other factors), vary between tebuconazole and prothioconazole. Potential differences in these properties between these active ingredients could have implications for FHB and DON management with tebuconazole + prothioconazole (Prosaro 421 SC), as well as sister products such as tebuconazole (Folicur 3.6 F) and prothioconazole (Proline 480 SC), since previous studies have shown that they vary in efficacy against FHB and DON (22).

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