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Assessment of the biological efficacy of chemical defense to prevent quality and yield loss due to Fusarium

Kémiai védekezési megoldások biológiai hatékonyságának értékelése kalászfuzáriózis ellen

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ABSTRACT

Fusarium head blight poses the most significant threat to the yield and quality of autumn cereals. Currently, there are no known completely resistant varieties, but distinctions can be made regarding resistance among different cultivars and hybrids. The chemical agents approved in Hungary can offer adequate protection against Fusarium head blight; however, the precise positioning of these pesticides is crucial for achieving optimal effectiveness. In both livestock feed production and human food processing, it is essential to produce toxin-free raw material. To achieve this, it is imperative to time pesticide applications considering weather conditions. This study assessed the influence of weather conditions on the proliferation of an artificially introduced Fusarium strain and its DON toxin production. Additionally, the impact of microclimatic factors on the development of natural infections was examined. Furthermore, the extent to which the chemical pesticides used in the study can reduce DON toxin levels was measured, based on whether they were applied before or after the introduction of Fusarium inoculum. According to the main observations, the toxin content is significantly higher at the later time of inoculation. In general, azole and strobilurin-type fungicides achieved excellent efficiency.

Keywords: artificial inoculation, deoxynivalenol mycotoxin (DON), fungicide efficacy, *Fusarium graminearum*, winter wheat

ÖSSZEFOGLALÁS

A kalászfuzáriózis az őszi kalászosaink termés mennyiségét és minőségét leginkább veszélyeztető megbetegedés. Jelenleg rezisztens fajta nem létezik, az ellenállóság tekintetében viszont különbséget tudunk tenni a fajták és a hibrideket illetően. A jelenleg Magyarországon engedélyezett kémiai hatóanyagok megfelelő védelmet képesek nyújtani a kalászfuzáriózis ellen, viszont a növényvédőszerek pozícionálása rendkívül fontos a jó hatékonyság elérése érdekében.

Az állati takarmányozás és a humán élelmiszer előállítás alapfeltétele, hogy DON toxin mentes terményt állítsunk elő, ehhez viszont az időjárási körülmények figyelembevételével tudnunk kell időzíteni a növényvédőszeres kezeléseket. A vizsgálatunkban megmértük, hogy az időjárási körülmények milyen hatást gyakoroltak a mesterségesen kijuttatott Fuzárium törzsünk felszaporodására és DON toxin termelésére, illetve a természetes fertőzés kialakulásában milyen hatást gyakorolt a mikorklimatikus tényező. Továbbá megmértük, hogy a vizsgálatban szereplő kémiai növényvédőszerek milyen mértékben képesek csökkentetni a DON toxin szinteket azalapján, hogy a fuzárium inókulum kijuttatás előtt vagy után juttatjuk azt ki.

Kulcsszavak: mesterséges fertőzés, deoxynivalenol mikotoxin (DON), gombaölőszer hatékonyság, *Fusarium graminearum*, őszi búza

INTRODUCTION

Wheat contributes significantly to the global economy, being the second most produced cereal globally. Crops are constantly attacked by pests and pathogens and compete with weeds, leading to significant yield losses (da Luz et al., 2017). *Fusarium* head blight (FHB) is a devastating disease of wheat and other small grain crops. The main cause of FHB in the United States is *Fusarium graminearum* Schwabe (Bockus et al., 2010). Still, other causes are also clustered within the *Fusarium graminearum* species complex (Aoki et al., 2012). FHB causes economic losses not only in terms of yield and reduced grain volume but also due to the accumulation of the deoxynivalenol (DON, vomitoxin), nivalenol and its acetylated derivatives and zearalenone (ZEN) mycotoxin in grain (Huang et al., 2023; Sherif et al., 2023).

A significant part of the fungicide used is applied in response to the presence of the disease rather than to the imminent (either detected or predicted) risk of the disease. This often leads to unsatisfactory control and the feeling that the fungicides do not work properly. Thomas (1986) pointed out that some plants that receive up to three poorly timed sprays often suffer as much disease as untreated plants, suggesting that some fungicides are applied too late for effective disease control.

According to the estimates of Polley and Thomas (1991), fungicides used to control foliar diseases of winter wheat account for more than 20% of the variable cost of wheat production and are an integral part of ear crop management.

There is also growing concern that sublethal doses of some fungicides may lead to increased mycotoxin production by *Fusarium* species (Milus and Parson, 1994; D'Mello et al., 1998). Such effects have been observed in several in vitro studies with some compounds that inhibit the growth of *Fusarium* species but increase trichothecene production (Moss and Frank, 1985; Placinta et al., 1996; D'Mello et al. (1997).

The severity of FHB is increased by heavy rainfall during flowering and grain filling of wheat (Lacey et al., 1999; Jenkinson and Parry, 1994).

The most effective means of protection against FHB is the use of resistant varieties. The success of breeding programs targeting resistant genotypes depends to a large extent on the availability of resistant germplasm, the genetic diversity of breeding populations, and the method of reliable estimation of the level of resistance in breeding lines, which enables the efficient selection of advanced individuals (Steiner et al., 2017).

Besides chemical control against *Fusarium* head blight, several research groups are also involved in breeding for resistance. Based on their findings, it can be stated that all varieties found in commercial cultivation are susceptible to *Fusarium*, thus further breeding efforts are needed to develop *Fusarium*-resistant varieties (Murashko et al., 2022; Demydov et al., 2024)

Research has shown that FHB also affects the use of seeds for planting purposes, as the *Fusarium* infection of wheat seeds can result in significant seedling mortality without fungicidal seed treatment, and different seed treatments vary in their ability to reduce these seedling losses (Sooväli et al., 2017).

Under favorable environmental conditions, the majority of *Fusarium* species can produce various toxic secondary metabolites, e.g. trichothecenes, zearalenone and moniliformin. The occurrence of such natural contaminants (mycotoxins) in cereals is of great concern, as their presence in feed and food is often associated with chronic or acute mycotoxicoses in livestock and may pose a threat to human health (Bottalico and Peronne 2002; Visconti, 2001). Deoxynivalenol (DON) is a trichothecene mycotoxin produced primarily by *F. graminearum* and *F. culmorum*, and most commonly associated with *F. culmorum*. ins. (Bai et al., 2001; Visconti et al., 1986). In most parts of the world, several fungicides have been tested for their effectiveness in reducing FHB in wheat. According to Haidukowski et al. (2005), the use of certain fungicides reduced disease severity and mycotoxin contamination of infected grains by 77% and 89%, respectively. Hudec et al. (2019) found in Slovakia that chemical fungicides reduced the severity of FHB by 60.2-75.3% in 2011 and by 71.6-85.5% in 2012.

MATERIALS AND METHODS

Our experiments were carried out in 2022 at the Research facility of KITE Zrt. in Derecske, Hungary (latitude 47°22'15.N, longitude 21°33'03.S, elevation 95 m). The area receives an average annual precipitation of 550 mm. The research facility is located in an intensively cultivated agricultural area typical of the Hajdúság region.

The area was a well-cultivated arable land with chernozem classified as KA31 (sandy loam). The average soil pH in KCl was 6.8 (neutral), and the humus content was 1.6% (low). The original available phosphorus content (P_2O_5) in the soil was 305 mg/kg (very good), and the available potassium content (K_2O) was 255 mg/kg (good) in dry soil.

Crop technology

In 2021, the precrop on the field was watermelon (*Citrullus lanatus*). The fertilizer was applied once in the form of a liquid UAN solution with a specific active ingredient content of 100 kg/ha. Lindbergh autumn wheat variety was used, sown at a rate of 4 million seeds per ha at a depth of 5 cm. The sowing date was October 15, 2021.

Experimental setup

In the experiment 160 small plots were established, each plot measuring 2.5 m x 10 m (25 m^2). The experiment was divided into two blocks: the first block (80 plots) received artificial inoculation at the beginning of flowering (BBCH 60–61), while the second block received artificial inoculation during mid-flowering (BBCH 64–65) (Figure 1).

Each block was further divided into two parts. In the first block, 80 small plots were created, where 40 plots were sprayed with 9 different chemical pesticides (Table 1) against *Fusarium* head blight at the end of heading and the beginning of flowering (BBCH 59–60), and another 40 plots were sprayed at the beginning of flowering (BBCH 60–61).

Similarly, in the second block, 80 plots were established, with 40 plots sprayed at the beginning of flowering (BBCH 62–63) and the other 40 plots treated after mid-flowering (BBCH 66–67) with the same 9 chemical pesticides (Table 1) against *Fusarium* head blight (Figure 1).

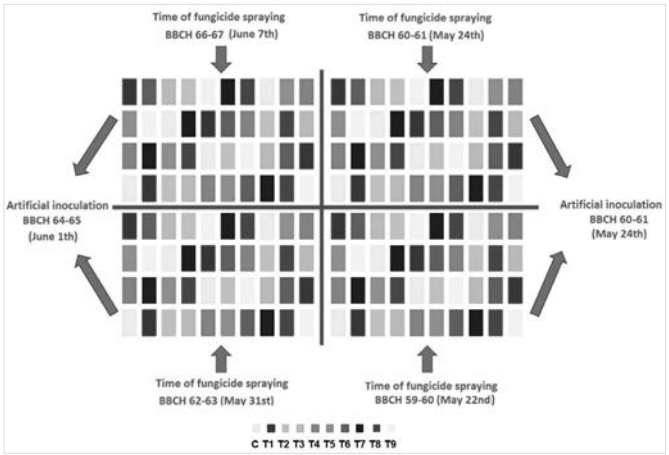


Figure 1. The arrangement of the experimental plots

For the artificial inoculation an identified strain (O'Donnell et al., 2004), *Fusarium graminearum* Schwabe F.00970 was purchased as liofilzed sample from the National Collection of Agricultural and Industrial Micro-organisms (NCAIM, Hungary).

Table 1. The treatments used in the experiment

Code	Treatment
C	Control (no treatment)
T1	250 g/l azoxystrobin + adj. (0.8 l/ha) + 300 g/l prothioconazole + adj. (0.5 l/ha)
T2	150 g/l prothioconazole, 75 g/l benzovindiflupyr + adj. (1 l/ha)
T3	150 g/l trifloxystrobin, 175 g/l prothioconazole + adj. (1 l/ha)
T4	160 g/l prothioconazole, 300 g/l spiroxamine + adj. (1.25 l/ha)
T5	125 g/l prothioconazole, 125 g/l tebuconazole + adj. (1 l/ha)
T6	130 g/l prothioconazole, 65 g/l bixafen, 65 g/l fluopyram + adj. (1.5 l/ ha)
T7	250 g/l prothioconazole + adj. (0.75 l/ha) + 100 g/l mefentrifluconazole + adj. (0.75 l/ha)
T8	100 g/l pyraclostrobin, 100 g/l mefentriflucanazole + adj. (1.2 l/ha)
T9	100 g/l mefentriflucanazole, 150 g/l kresoxim-methyl + adj. (1 l/ha)

The identifiers of the strain in other collections are NRRL 5883, ATCC 46779; CBS 110261, and MAFF 237812. The sample was activated following the recommended method of the NCAIM. The conidium suspension was produced in two rounds of culturing in potato dexrose broth (PDB, Merck, Germany) media. The breeding was conducted in 100 ml of liquid media in 300 ml Erlenmeyer flasks with Unitwist 400 orbital shaker (180 r x min^{-1}) at room temperature (20 ± 3 °C). The activation and the culturing were conducted under sterile conditions. The coniduim suspension was diluted to 6x105 conidia x ml^{-1} before the inoculation with 20 °C tap water.

Within each plot, a 2.5 m^2 area at the center was designated, and the crop was artificially infected with *F. graminearum* inoculum. The inoculation was carried out in the evening hours. Using a hand sprayer (Daewoo DAPSP5L), we sprayed the already marked 2.5 m^2 area in the center of each plot evenly. The crop was irrigated with a micro-sprinkler irrigation system (Figure 4), applying 4–5 mm of water every day from the flag leaf emergence to the beginning of milk ripening (BBCH 47–70). The precipitation distribution in 2022 deviated from the years of 1952–1990 average. The necessary rainfall for the development of a natural *Fusarium* epidemic did not occur in May and June. There was a significant drought in the region during this period (Figure 2).

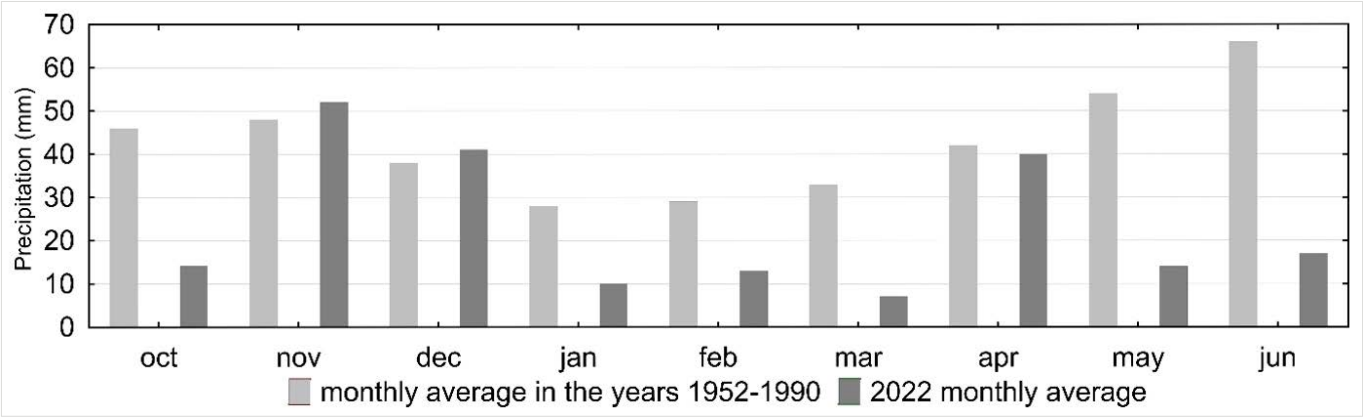


Figure 2. Precipitation distribution during the growing season

The *Fusarium* inoculation took place in the second block on June 1, 2022, following the irrigation of the crop in the evening hours. The fungicides were applied on May 31, 2022 (preventively), and on June 7, 2022 (Figure 3).

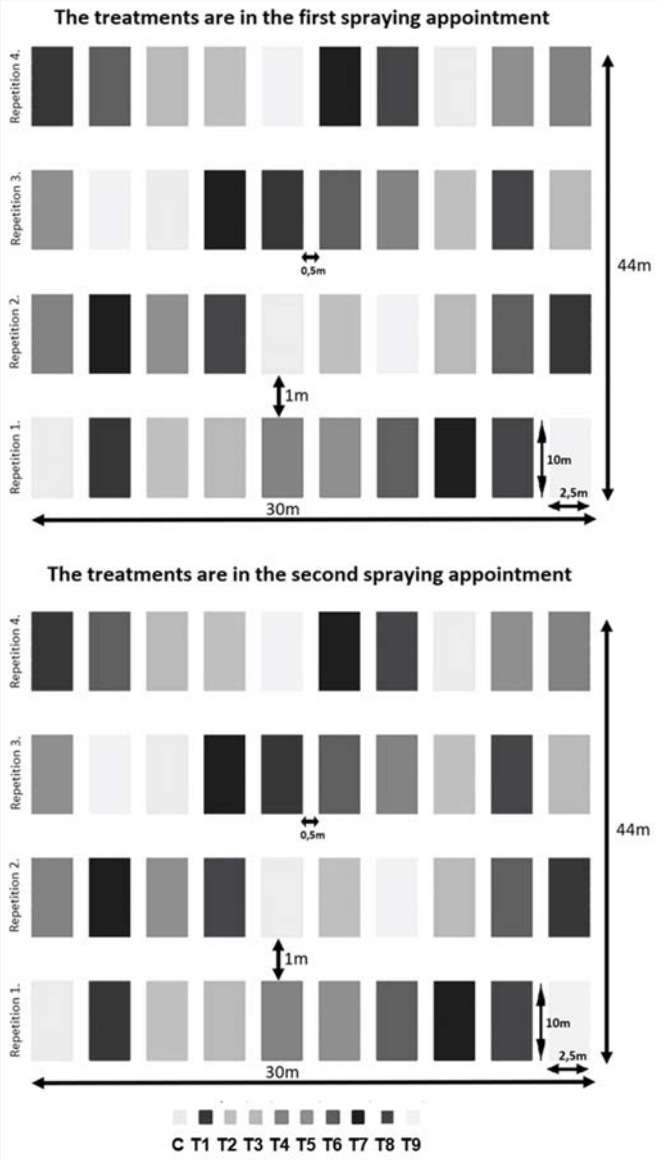


Figure 3. Experimental design

Data collection

Before harvesting, two subplots, each measuring 2.5 m^2, were designated within each (25 m^2) plot. These designated areas were harvested using a plot combine harvester (Zürn 130se). The weight of the collected samples was measured (T-kale MCW-150M), and they were stored in paper bags until the DON toxin content analysis. An automated weather monitoring station (CSMEWS.03) was set up at the edge of the field to record air temperature, humidity (SHT35), wind speed (DAVIS 7911), and leaf surface moisture duration during the growing season (Developed in-house by Csiha Ltd.).

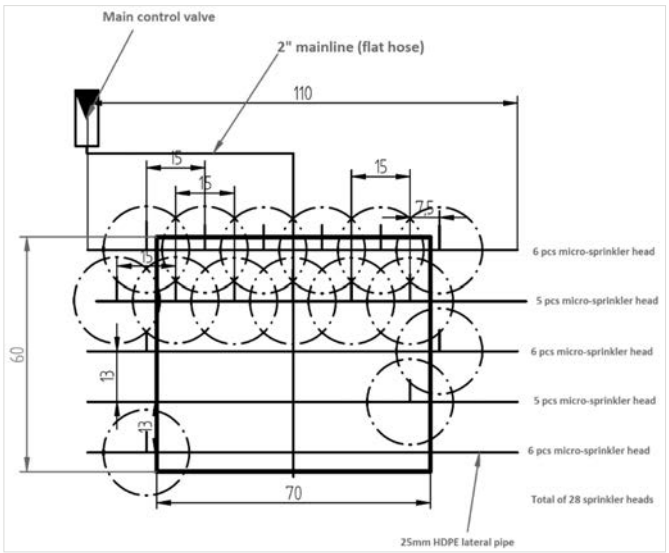


Figure 4. Schematic of the Irrigation System Layout

Analysis of DON

Wheat samples were milled with Perten laboratory mill 3310 (Perkin Elmer Inc., Waltham, MA, USA). Five grams of milled sample was extracted with 20 mL of MeCN/H_2O (60/40, v/v) in a 50 mL centrifuge tube using an overhead shaker for 2 hours and was subsequently centrifuged at 10000 rpm for 10 min. Before and after shaking, samples were sonicated for 10 min in an ultrasonic bath. The supernatant was pipetted into an HPLC autosampler vial. DON was quantified by LC-MS applying the following conditions. Samples were analyzed using an Agilent 1100 high-performance liquid chromatograp (HPLC; Santa Clara, CA, USA) connected to an Agilent 1946D mass spectrometer (MS) equipped with an electrospray ion source (ESI). The HPLC system consisted of a vacuum degasser, a binary pump, a well plate sampler, and a column thermostat set to 40 °C. Gradient separation was performed on a Security Guard column (C18; Phenomenex, Torrance, CA, USA) and a Kinetex C18 (50 × 4.6 mm, 2.6 µm; Phenomenex) analytical column at a flow rate of 800 µL/min. Both

solvent A (water) and solvent B (acetonitrile) contained 0.1% (v/v) formic acid. All three chemicals were HPLC-MS grade and were purchased from VWR International Kft. (Debrecen, HU). The gradient started with 0% B and increased linearly to 48.6 % in 4 min, then increased to 79% in 2 min, and further increased to 100% in 0.2 min, held it for 2 min and returned to the initial value in 2.3 min followed by holding this value for 3 min. The flow rate of the analysis was as follows: started with 800 µL /min, held it for 6.6 min, increased to 2000 µL/min in 0.4 min held it to 1.4 min then returned to 800 µL/min in 1.4 min. The analysis was started with an injector program, including the addition of 5 ng /µl Biopure ^{13}C isotope fully labelled deoxynivalenol internal standard (Romer labs Ltd, Getzersdorf, AT) to each 4 µl sample. The mass spectrometer (MS) was set to the following ESI parameters: nebulizer gas (N_2) pressure, 50 psi; drying gas (N_2) flow rate, 12 L/min and temperature, 350 °C; capillary high voltage, 3000 V. MS data was acquired in positive ion SIM (selective ion monitoring) mode: 2–4 min: *m/z* 297 and 312. Stock solutions for calibration were freshly prepared from vials containing 100 µg DON (Fumizol Ltd. Szeged, HU) by dissolution with 1 ml blank sample which did not contain DON toxin (based on the sample preparation described above). The following calibration points were diluted from the stock solution with the same solvent: 10, 5, 2.5, 1.125, 0.625, 0.3125, 0.039075 ng/µl. The limit of detection (LOD) and limit of quantification (LOQ) values were established as the lowest detectable and quantifiable concentration level of calibration samples with the suitable signal-to-noise ratio (3:1 for LOD and 10:1 for LOQ). LOD and LOQ of DON were as follow: LOD: 0.039 ng/µl, LOQ: 0.078125 ng/µl, which calculated on the weight of extracted wheat were: LOD: 156.3 µg/kg LOQ: 312.5 µg/kg The linearity of the DON calibration curve was: 0.07875–2,5 ng/µl (312.5–10000 µg/kg), R^2: 0.9991. The results have been evaluated by isotope-labelled internal standard calibration.

Statistical analysis

The evaluation of the effectiveness of chemical fungicides was based on DON toxin levels and crop yield. Data from artificially infected plots and non-infected plots were separately evaluated. The assumptions for parametric tests as the homogeneity of variances and the normal distribution were checked with Levene's test and Q-Q plot diagrams respectively. As our data did not meet the assumptions for most cases, Kruskal-Wallis non-parametric tests were used and backed them up with Mann-Whitney tests as pairwise comparisons. The statistical analysis was conducted using IBM SPSS 28.0.1.0 statistical software.

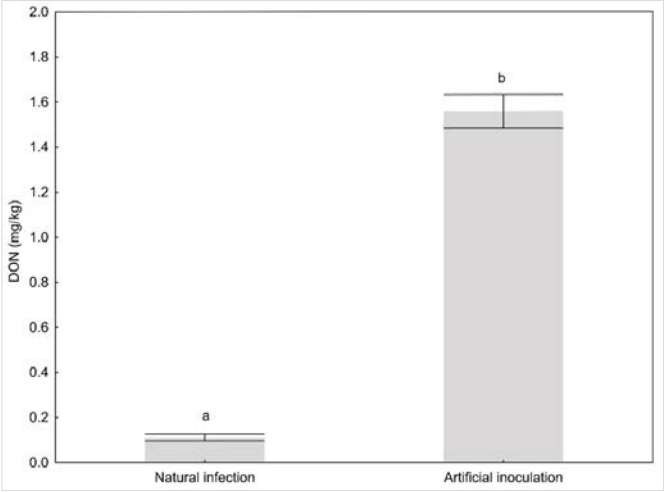
RESULTS AND DISCUSSION

Winter wheat is one of the most important and widely grown crops in the world, and an important source of protein and calories for human nutrition. Ensuring plant health during development and producing a toxin-free crop for later use is of primary importance. Previous research has found that there are several different strategies for reducing toxin levels in crops and processed food end products, including pre-harvest, post-harvest, and processing methods (Torres et al., 2019). As wheat is most susceptible to *Fusarium* head blight during flowering, chemical control can also be applied at this time (Sutton, 1982). Also, these control strategies with different fungicides can be useful if application technology and timing are appropriate. Among the two inoculation dates included in the study, the earlier one (May 24) did not result in proper infection, hence the DON toxin content was also low. Based on the measured values, we could not detect a statistically significant difference compared to the control plot. The low mycotoxin contamination occurred because the weather conditions during the time of artificial infection were not ideal for *F. graminearum*, particularly the leaf surface moisture (which was only 30 minutes) (Table 2). According to the literature, 7–8 hours of leaf surface moisture is needed for *F. graminearum* to infect the crop.

Table 2. The variation of weather factors during the time of artificial inoculation

	Date	Weather conditions				
		Air temperature (°C)	Relative humidity (%)	Leaf surface moisture (minutes)	Plant phenology (BBCH)	Wind speed (m/s)
First artificial inoculation	May 23rd	15.7	66	0	61	1.4
	May 24th	14.8	79	30	61	2
	May 25th	19.1	83	350	62	1.4
	May 26th	19.2	63	135	62	2.2
Second artificial inoculation	May 31st	16.5	86	0	65	0.8
	June 1st	17.5	87	445	65	0.8
	June 2nd	19.5	75	550	66	0.8
	June 3rd	21.2	71	169	66	1.2

At the time of the second artificial inoculation (June 1), the weather conditions were suitable for *F. graminearum* to infect the cereal crop (Table 2). Based on the overall results of the investigations conducted during the second artificial inoculation, it can be concluded that there were statistically significant differences between the artificial inoculation and naturally infected control plots (Figure 5). Based on the results of Scarpino et al. (2015) and Machado et al. (2017) azole fungicides have been shown to be effective against the pathogen, and similarly several of these agents in our present study resulted in significant reductions in toxin levels. Nevertheless, in addition to the appropriate growing environment and the use of resistant cultivars, the active ingredient composition of treatments and the timing of application both determine the success of chemical control (Yoshida et al., 2012; Pirgozliev et al., 2008; Tateshi et al., 2014), as fungicide efficacy can vary from year to year and between production areas even when standard spraying methods are followed (Šíp et al., 2010; Paul et al., 2007). In our study significant differences were measured between the two application timings, resulting in the second application being more effective in many active substances. These results confirm the findings of Yoshida et al. (2012), where late fungicide application 20 days after flowering resulted in significantly lower toxin levels.



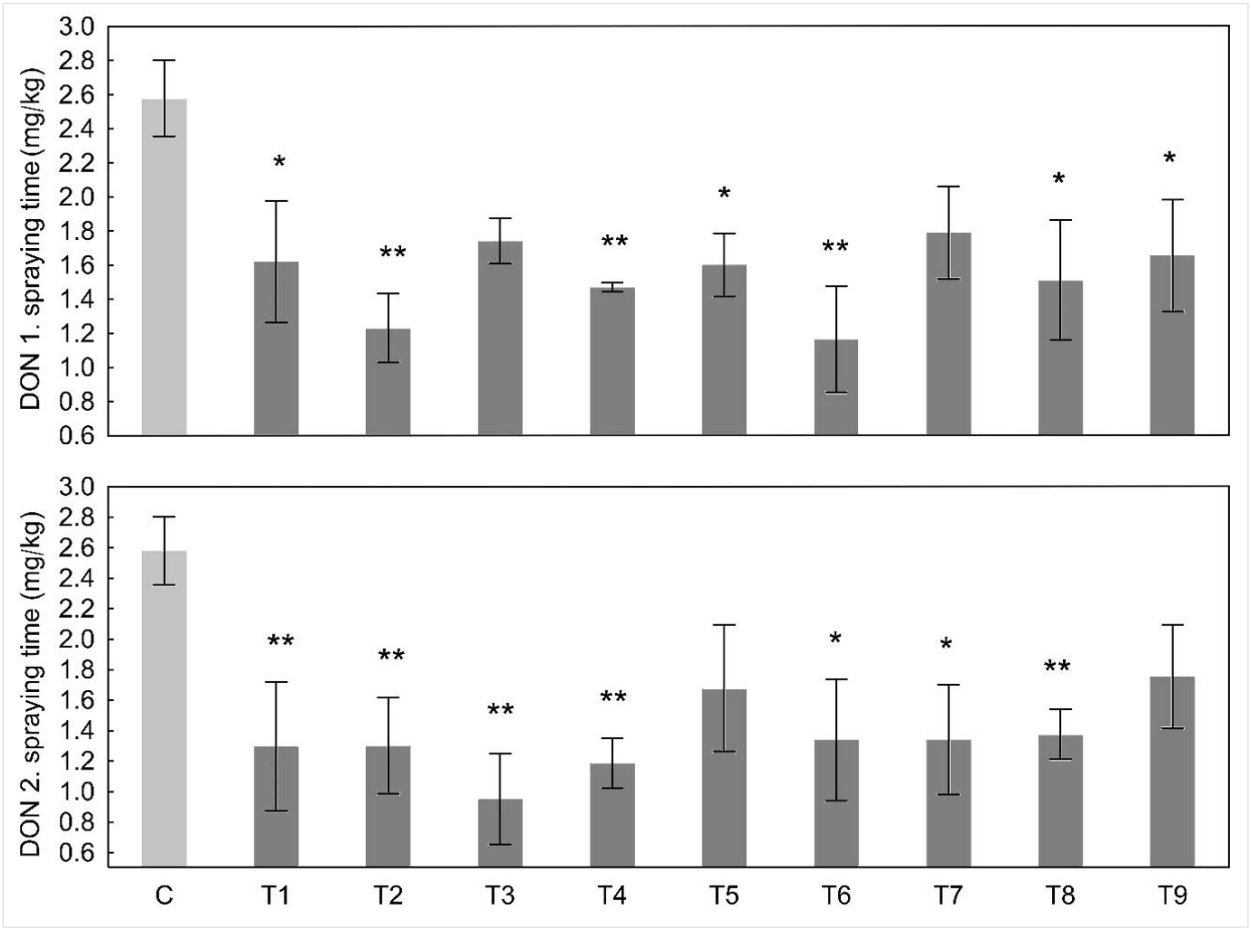
Lowercase letters indicate significant differences according to the Mann-Whitney U test ($P < 0.05$)

Figure 5. The DON toxin level (mg/kg; ± SE) based on samples from naturally infected and artificially inoculated control plots (artificial inoculation on June 1st)

Mann-Whitney non-parametric tests revealed that the pre- (May 31) and post-inoculation (June 7) sprayed fungicides had different effects on DON toxin production by the *F. graminearum* strain used in the experiment (Figure 6). Among the fungicides applied at the first spraying (May 31, 2022) the treatments 150 g/l trifloxystrobin, 175 g/l prothioconazole+adj. (1 l/ha) (T3) and 250 g/l prothioconazole+adj. (0.75 l/ha) + 100 g/l mefentrifluconazole+adj. (0.75 l/ha) (T7) did not show significant differences (Mann-

Whitney U: $P > 0.05$) compared to the control. Treatments 250 g/l azoxystrobin+adj. (0.8 l/ha) + 300 g/l prothioconazole+adj. (0.5 l/ha) (T1), 125 g/l prothioconazole, 125 g/l tebuconazole+adj. (1 l/ha) (T5), 100 g/l pyraclostrobin, 100 g/l mefentrifluconazole+adj. (1.2 l/ha) (T8), and 100 g/l mefentrifluconazole, 150 g/l cresoxymethyl+adj. (1 l/ha) (T9) showed significant differences at Mann-Whitney U: $0.01 < P < 0.05$ level compared to the control, while treatments 150 g/l prothioconazole, 75 g/l benzovindiflupyr+adj. (1 l/ha) (T2), 160 g/l prothioconazole, 300 g/l spiroxamin+adj. (1.25 l/ha) (T4), and 130 g/l prothioconazole, 65 g/l bixafen, 65 g/l fluopyram+adj. (1.5 l/ha) (T6) showed significant differences at Mann-Whitney U: $P < 0.01$ level (Figure 6). At the 1st spraying date, Kruskal-Wallis H = 18.07, $P = 0.034$

The effectiveness of fungicides applied in the second spraying (June 7, 2022) differed; treatments 125 g/l prothioconazole, 125 g/l tebuconazole+adj. (1 l/ha) (T5) and 100 g/l mefentrifluconazole, 150 g/l cresoxymethyl+adj. (1 l/ha) (T9) showed no significant differences (Mann-Whitney U: $P < 0.05$). Treatments 130 g/l prothioconazole, 65 g/l bixafen, 65 g/l fluopyram+adj. (1.5 l/ha) (T6) and 250 g/l prothioconazole+adj. (0.75 l/ha) + 100 g/l mefentrifluconazole+adj. (0.75 l/ha) (T7) had significant differences at Mann-Whitney U: $0.01 < P < 0.05$ level compared to the control. Treatments 250 g/l azoxystrobin+adj. (0.8 l/ha) + 300 g/l prothioconazole+adj. (0.5 l/ha) (T1), 150 g/l prothioconazole, 75 g/l benzovindiflupyr+adj. (1 l/ha) (T2), 150 g/l trifloxystrobin, 175 g/l prothioconazole+adj. (1 l/ha) (T3), and 160 g/l prothioconazole, 300 g/l spiroxamin+adj. (1.25 l/ha) (T4)



The asterisks indicate the statistical differences compared to the control based on the Mann-Whitney U-test (*$0.01 < P < 0.05$; **$P < 0.01$). At the 2nd spraying date, Kruskal-Wallis H = 17.50; $P = 0.041$

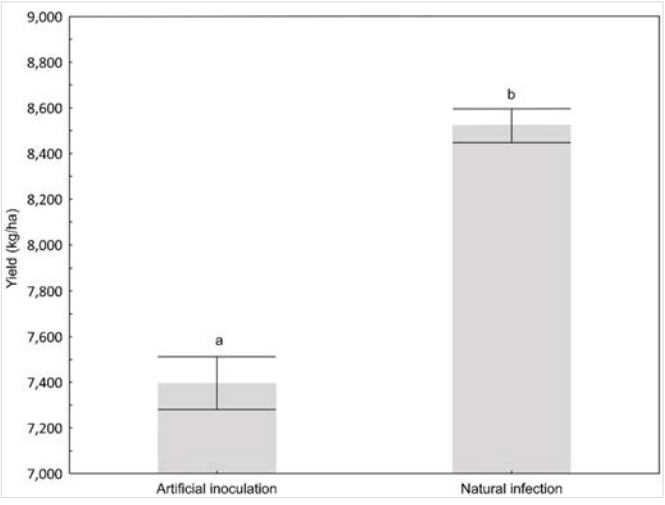
Figure 6. The DON toxin levels (mg/kg; ± SE) for each treatment at the second artificial inoculation (June 1) time point, according to the fungicide spraying timings

showed significant differences at Mann-Whitney U: $P > 0.01$ level compared to the control (Figure 6).

Fungicides belonging to the demethylation inhibitory (DMI) class are widely used to control FHB and DON contamination of cereal plants (Wegulo et al., 2015; McMullen et al., 2012). In a study by Paul et al. (2018) the application of DMI fungicides to wheat anthers at the Feekes 10.5.1 growth stage was the FONH Thinness Index and the most effective treatment. Azole fungicides have been reported to be the most effective agents for controlling FHB and reducing major mycotoxins such as deoxynivalenol (DON) in cereals (Scarpino et al., 2015). Our research findings stand in accordance with these previous results, as T2 (prothioconazole + benzovindiflupyr), T6 (prothioconazole + bixafen + fluopyram) and T3 (prothioconazole + trifloxystrobin) resulted in the highest level decrease of DON toxin from the results of the first and the second artificial inoculation time points, respectively.

Changing the spraying time did not significantly impact the effectiveness of either fungicide.

Analyzing the crop yields, it can be concluded that the plots subjected to artificial inoculation on June 1st achieved significantly lower yields ($P < 0.05$) (Figure 7).



Lowercase letters indicate significant differences according to the Mann-Whitney U test ($P < 0.05$)

Figure 7. The crop yield level (kg/ha; ± SE) based on samples from naturally infected and artificially inoculated control plots (artificial inoculation on 06.01)

Besides the changes in toxin levels, *F. graminearum* also had a significant impact on the crop quantity. The infection also affects Yield quantity and quality, causing significant yield losses and lower quality (Salgado et al., 2011). Our results expressed the higher effect of artificial inoculation on yield, even though the weather conditions were optimal for natural infection. Similar results were found in previous research, confirming the global importance of the pathogen and the plant protection against it (Blandino et al., 2006; Haidukowski et al., 2005; Salgado et al., 2015). Overall, the application of technology of chemicals to reduce crop losses is the main aim of crop protection.

CONCLUSIONS

In the application of chemical fungicides containing active ingredients against *Fusarium* head blight, we observed that the timing of application significantly influences the product's ability to reduce the level of DON toxin production. Inoculation performed at a later time (middle of flowering) led to higher toxin contamination in all. Products applied after artificial inoculation generally had a better impact on reducing the DON toxin production by *Fusarium* compared to those applied before inoculation. The most effective results were achieved with products belonging to the strobilurin and azole chemical groups.

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