

Assessment of in-crop treatments aimed at reducing mycotoxin contamination of maize primarily against *Ostrinia nubilalis* Hbn. (Lep. Crambidae), in addition to diverse tillage systems

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Abstract. Our field studies aimed to assess the effect of different tillage systems (conservation and conventional tillage) and a combination of unauthorized insecticide in maize (cyantraniliprole) and a fungicide (tebuconazole, prothioconazole) on the plant health status of maize in a maize monoculture stand. The effects of these treatments were investigated under large plot conditions by assessing and analysing satellite-based NDVI images, the in-vegetation damage of the dominant chewing pests of maize (*Diabrotica v. virgifera*, *Ostrinia nubilalis*, *Helicoverpa armigera*) and the HPLC-based toxin content (ZEA, DON, FB1, FB2) of plant samples from the different treatments at the end of the vegetation. The analysis of NDVI records confirmed that maize stands under conventional tillage have lower photosynthetic activity during the growing season, and plant stress occurs due to water stress. Consequently, the percentage of mycotoxins tested was higher in such a stand. Due to its insecticidal absorption effect, the analysed cyantraniliprole caused a prolonged suppression of the damage caused by *O. nubilalis*, the primary pest and the pathway for toxin-producing fungi. Fungicidal sprays timed to the peak of tasseling with the active ingredient tebuconazole+prothioconazole reduced toxin levels under detection limits in plant samples. Our results justify targeted insecticidal and fungicidal treatments in extensive maize stands.

Keywords: cyantraniliprole, damage patterns, fusariotoxins contamination, HPLC, in-crop treatment, NDVI, *Ostrinia nubilalis*, triazole fungicide

Introduction

The most severe phytosanitary concern of today's harvested cereal and, thus, maize items is the colonisation of various toxin-producing microfungi, which carries the possibility of further animal and human illness in itself (Nelson et al. 1981, Deshpande 2002, Waśkiewicz & Goliński 2013, Yu & Pedroso 2023). The increase in their occurrence in the last decade is due, among other things, to the rise in extreme weather phenomena escorting global climate change and indirectly to the promotion of pest organisms (Farkas et al. 2013, Chavda et al. 2014). These mycotoxins-producing microfungi successfully attack weakened, stressed plants. Among the species of the notorious genus *Fusarium*, members of the so-called liseola section (*Fusarium moniliforme*, *F. moniliforme* var. *subglutinans*, *F. polyferatum*) are typical wound parasites (Torres et al. 2001). The dangerous toxins produced by them are zearalenone (F-2 toxin, ZEA), trichothecenes [T-2 toxin, HT-2 toxin, nivalenol (NIV), deoxynivalenol (DON), diacetoxyscirpenol (DAS), fusarenone-X (FX)] and fumonisins (FB1 - FB6) (Nesic et al. 2013). Other important mycotoxin-producing species are *Aspergillus flavus*, *A. parasiticus*, *A. nomius* and *A. ochraceus*, as well as black *Aspergillus* species (e.g. *A. carbonarius*, *A. niger*), which also produce aflatoxins and ochratoxins that cause severe diseases (Britzi et al. 2013). These growing health concerns have forced policymakers to set maximum tolerances for certain mycotoxins in feed rations through various regulations. Specifically, the EU Directive (2006) sets maximum toxin levels in maize and cereals' feed rations. This measure was

extended in 2007 to cover additional toxins [DON: 1.25 ppm (g t⁻¹ crop), zearalenone (ZON): 0.2 ppm, fumonisin: 2 ppm]. In the last decade, conservation tillage has also become increasingly widespread in Central Europe, and in dense crops, the practice of no-tillage has become more and more dominant (Derpsch 2008, Pires et al. 2017). In maize cultivation, the practice of mulch, cover cropping or other strip-tillage systems is also nowadays emerging in different parts of Central Europe. Of course, the different tillage systems used essentially determine the soil layer's soil porosity, moisture, and humus content (Mathew et al. 2012). However, the intervention in the soil as a complex system also has a decisive influence on the activity, occurrence, and even the reproduction of pest organisms associated with the soil as a medium. Maize stalks left on the surface from the previous year may provide an opportunity for *Fusarium* species to infest the following year (Behnke et al. 2021) or may serve as an excellent overwintering site for the European corn borer (*Ostrinia nubilalis* Hbn.) (Lep.: Crambidae), which is the primary transmitter (through the plant wounds formation) for colonization of mycotoxin-producing fungi in the cereal crops (Keszthelyi et al. 2021).

The reduction of mycotoxin contamination of the fields can be expected from the use of the appropriate soil cultivation system and from the use of insecticides that promote the colonization of chewing insects, as well as a targeted fungicide control (Alma et al. 2005, Folcher et al. 2012). In the last decade, *O. nubilalis* control has spread in extensive maize production (Keszthelyi & Pónya 2019). For these in-crop treatments, pyrethroids (cypermethrin,

deltamethrin, lambda-cyhalothrin), neonicotinoid (acetamiprid), *Bt*-toxin analogs, and diamide (chlorantraniliprole) insecticides belonging to the active substance group are used, which have weak translocation and whose efficacy depends above all on accurate prediction of the emergence of newly hatched *O. nubilalis* larvae (Bažok et al. 2009). The effectiveness of these treatments could be significantly enhanced by a more absorbable compound that translocates well in the plant, such as cyantraniliprole belonging to diamide, which is approved for use in the protection of other crops.

Another option for reducing mycotoxins in vegetation is targeted fungicide spraying. Among the many triazole, prothioconazole, and tebuconazole active ingredients are ubiquitous, which, in addition to inhibiting the establishment of microfungi, also provide a greening effect for developing maize plants (Blandino et al. 2012, Ivanov & Harizanova 2023).

Our field tests aimed to assess mycotoxin contamination in the laboratory as a function of maize with different tillage systems (conventional and conservation) and in-crop treatments (fungicide, insecticide spraying). In addition, we were curious about the extent of the toxin-mitigating effect of fungicidal interventions carried out in extensive stands. We aimed to evaluate the decreasing level of *O. nubilalis* damage

due to treatments with cyantraniliprole by assessing specific damage patterns in maize plants.

Material and methods

Experimental field and treatments

A large plot field (8 ha) study was set up in maize monoculture stands in 2023. In two neighboring parcels (4-4 ha) located on the border of Barcs, Somogy County (GPS: 46°02'28.80 "N 17°41'34.75 "E), in which maize hybrid (DKC5206, FAO 420) was sown. In the experimental field, maize was also grown in the previous year as a monoculture. Soil disinfection (tefluthrin: 15 kg ha⁻¹) was applied to the selected area in all the years of the study to control the larval infestation of the western corn rootworm (*Diabrotica v. virgifera* LeConte). No other insecticidal intervention was applied to the experimental vegetation in the sample plots in addition to the treatments used as stand treatments.

The two adjacent plots, each 4-4 ha in size, were treated with different tillage systems (Figure 1). One plot of 4 ha was treated with conventional plowing, while the other plot of 4 ha was treated with conservation tillage (mulch leaving no tillage). In the experimental area, four treatments with different formulations of fungicides and insecticides were applied in vegetation using a self-propelled sprayer on 1-1 ha each. The treatments were timed within one week after the flight peak of *O. nubilalis* observed by the light trap. The treatments (T), which were applied with different formulations and their application dates, are shown in Table 1.

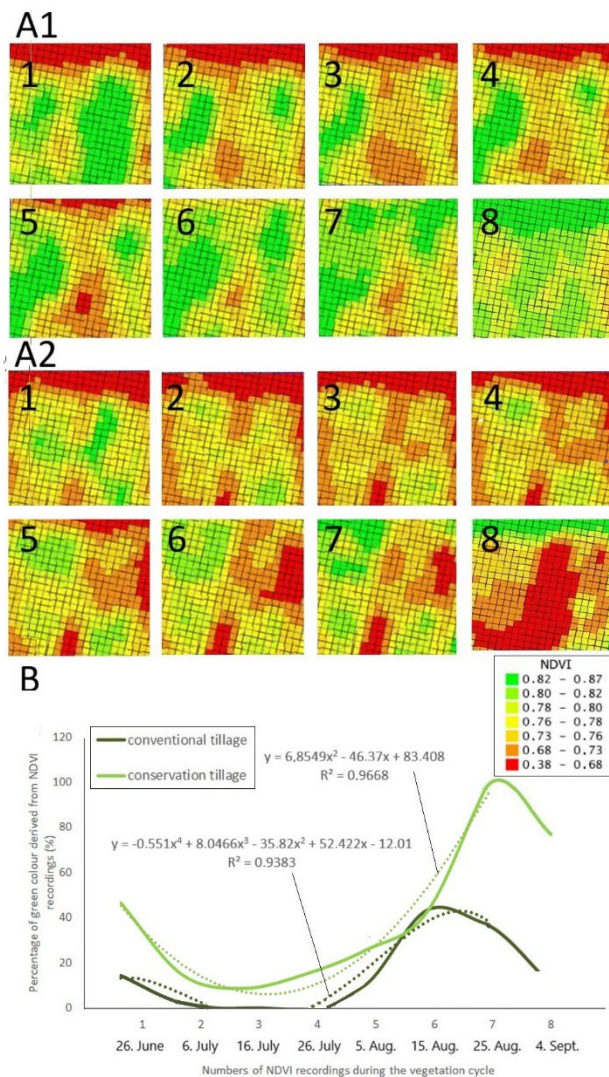


Figure 1. NDVI-based satellite images-series of experimental plots with different tillage systems (A1: conservation tillage; A2: conventional tillage), as well as the trend of vegetation greenness as a function of tillage (B)

Table 1. Composition and date of in-crop treatments (T) in the experimental maize plots in 2023

N°	treatments	active ingredients	dose	date
1.	fungicide	125 g/l prothioconazole - 125 g/l tebuconazole	1 l ha ⁻¹	
2.	insecticide	100 g/l cyantraniliprole	0.5 l ha ⁻¹	
3.	fungicide + insecticide	125 g/l prothioconazole - 125 g/l tebuconazole + 100 g/l cyantraniliprole	1 l ha ⁻¹ 0.5 l ha ⁻¹	23. June
4.	control	without in-crop treatment	-	

Table 2. Examined foliar damages and causative species in the experimental parcels (Eng. n = English name; Sci. n. = scientific name; T.ord. = taxonomical order; d.p. = damage patterns)

N°.	1.	2.	3.
Eng. n.	western corn rootworm	European corn borer	cotton bollworm
Sci. n.	<i>Diabrotica v. virgifera</i> LeConte, 1868	<i>Ostrinia nubilalis</i> Hbn. 1796	<i>Helicoverpa armigera</i> Hbn. 1805
T.ord.	Col.: Chrysomelidae	Lep.: Crambidae	Lep.: Noctuidae
d.p.	peeling of the leaf surface by adults	1. puncturing of the upper leaf by L ₁ stage larvae 2. broken tassels and stalks caused by burgeoning young larvae 3. hole and mastications on the stalk caused by boring larvae	mastication into the tassels or leaves caused by larvae

Determination of treatment-dependent mycotoxin content

Samples of 300 g were collected from each parcel immediately after harvest in 3 replicates per plot (24 samples in total). These representative samples from the experimental field were designed to contain maize vegetative (stalk, leaf) and generative (inflorescence, ear) organ pieces and parts.

The samples were taken to the HPLC laboratory of the Institute of Physiology and Nutrition, Kaposvár Campus, Hungarian University of Agricultural and Life Sciences. Here, the samples were first lyophilized, then ground to powder, then extracted with acetonitrile-water and finally, the extract was determined by high-pressure liquid chromatography coupled mass spectrometry (HPLC-MS) for the determination of the different toxin composition and amount. The direct measurement results were evaluated using Lab Solutions software, during which the toxin concentrations were also determined. The study aimed to quantify the fumonisins B1, B2 zearalenone (F-2 toxin, ZEA), and deoxynivalenol (DON) toxins in crop samples expressed in mg kg⁻¹.

Measured mycotoxin concentrations for the two groups were compared using an R-program (vertex 4.1.2) to detect any significant difference. The results were used to perform a risk assessment. The effect of different tillage and pesticide treatments on the fusariotoxin contents was statistically analyzed by two-way ANOVA ($p \leq 0.05$). The independent variables were the type of tillage (conventional and conservation tillage) and the different active ingredient compositions of the in-crop treatment. The dependent variable was the mycotoxin content.

Results

NDVI-based photosynthetic activity as a function of tillage system

NDVI imaging series of maize growing in experimental fields with different tillage (conventional, conservation tillage) at eight different time points are shown in Fig. 1. Even without analysis, it is clear that in the year 2023, the crop in the conventional tillage areas (A2) was much more exposed to climate-induced moisture deficit and high daily air

temperature values. Practically, by the end of the growing season (A2-8), the assimilation potential of the vegetation was minimized, and the appearance of arid patches with drying became significant. The percentage coverage of NDVI values 0.38-0.66 in the last satellite image of the plowed (conventional) parcel (4 September 2023) was precisely 43.361%.

In contrast, the maize field in the experimental parcel "greened back" after the initial drying out due to conservation tillage. The greenness of the crop increases after the A1-6 (15 August 2023) recording period and peaks in the 7th and 8th recording periods.

If we analyze the greenness trends indicating the photosynthetic activity of the stand (Fig. 1B), it can be seen that for both tillage types, the seasonal variation of NDVI-derived greenness values is parabolic, with trends described by polynomial equations. For both treatments, greenness declines significantly after maize tasselling and enters an upward phase by the end of August. However, the much stronger appearance of greenness values in the case of conservation tillage was demonstrated during the entire vegetation period analyzed, which was confirmed by the statistical analysis results. The variation of green color values with tillage extracted from NDVI-series of images of maize fields under different tillage systems showed statistically significant alteration with tillage according to the results of one-way ANOVA ($df=1$; $F=4.679$; $p=0.048$).

Insect damage formations depend on different compositions of in-crop treatment

The percentage of damage symptoms manifested in maize vegetation as a function of treatments is shown in Fig. 2. Overall, it is evident that a higher rate of insect damage appeared at the second inclusion (T+14d) after pesticide treatments, in comparing pests, *D. v. virgifera* damage dominated at both recording dates. Despite in-crop spraying

timed to the flight peak of *O. nubilalis*, damage occurred around 5-20% on day seven after treatment, independently of applied tillage systems. Both upper leaf punctures and broken tassels caused by *O. nubilalis* were observed. In the experimental plots, the most minor damage was observed to *H. armigera*, which is most affected during the last stage of generative part-formation and maturation. At this stage, it is most recognizable by leaf and stems damage, but its maize ear damage, which causes significant economic losses, has not yet developed.

The results of the statistical analysis of the effects of different soil and stand treatments on the expression of the examined pest damage maps are implied in Table 3. At the time of recording T+7d, the conventional tillage plots had the highest level of insect damage, which seemed to level off by T+14d. The effect of different tillage systems on damage development had a statistically significant impact on damage formation for almost all pests at both recording dates (except at the time of recording *D. v. virgifera*). In the case of *O. nubilalis* damages, the different tillage practices did not induce a statistically verifiable consequence.

All pesticide treatments (irrespective of the active ingredient) had a statistically significant effect on damage development at both observation time points. When comparing the different active ingredient treatments, insecticide treatments caused the most significant reduction in damage formation. The most spectacular reduction in

damage was recorded for insecticide treatments of *D. v. virgifera* on day seven after treatment. However, by day 14 after treatment, the damage caused by *D. v. virgifera*'s continuous emergence had significantly increased.

Toxin contamination in maize residue depends on different tillage and in-crop treatments

The projected quantities of maize residues in mg kg⁻¹ from different treatments can be seen in Fig. 3. The study confirmed that fumonisin B1 and B2 toxins were detectable in all treatment settings. Deoxynivalenol toxin was detected in laboratory samples in only four cases, three of them (conservation tillage + fungicide; conservation tillage + insecticide; conventional tillage + ins-fungus) just above the detection limit. In none of the samples did the recorded toxin contamination reach the maximum levels allowed for maize and maize-based products as defined by EC (2006). For both tillage systems, residues from parcels not treated with pesticides contained the highest toxins. High levels of fumonisin B1 toxin were recorded in samples from parcels with conventional tillage + untreated combination. As previously assumed, the most diverse and toxin-contaminated item was detected in conservation tillage + untreated plots samples. Here, in addition to fumonisin B1 and B2 toxins, zearalenone, and deoxynivalenol toxins were present at levels well above the limit of detection (LOD).

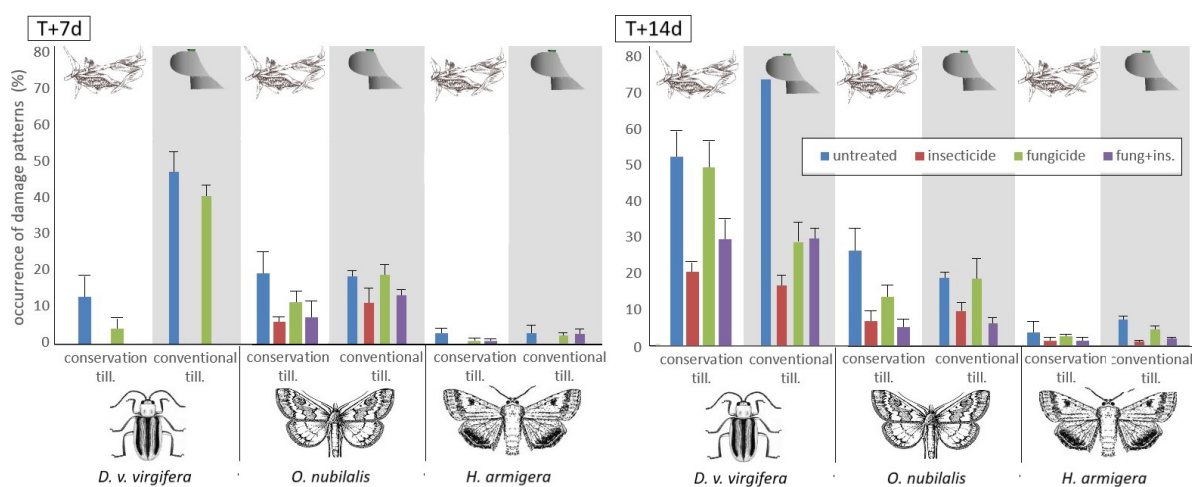


Figure 2. Percentage occurrence of damage patterns of chewing mouthpart pests in experimental maize parcels as a function of different treatments

Table 3. Results of statistical analysis of different treatments and damage patterns using two-way ANOVA ($p \leq 0.05$) (bold decimal fractions indicate that there is no statistical correlation)

	<i>D. v. virgifera</i>			<i>O. nubilalis</i>			<i>H. armigera</i>		
	df	F	<i>p</i>	df	F	<i>p</i>	df	F	<i>p</i>
T+7d									
pesticide composition	3	98.670	≤ 0.001	3	12.047	≤ 0.001	3	8.919	≤ 0.001
tillage system	1	140.829	≤ 0.001	1	13.674	≤ 0.001	1	11.126	0.002
interaction	3	45.965	≤ 0.001	3	1.921	0.146	3	1.563	0.217
T+14d									
pesticide composition	3	138.520	≤ 0.001	3	250.040	≤ 0.001	3	45.569	≤ 0.001
tillage system	1	0.061	0.805	1	37.101	≤ 0.001	1	39.516	≤ 0.001
interaction	3	29.582	≤ 0.001	3	2.705	0.061	3	8.290	≤ 0.001

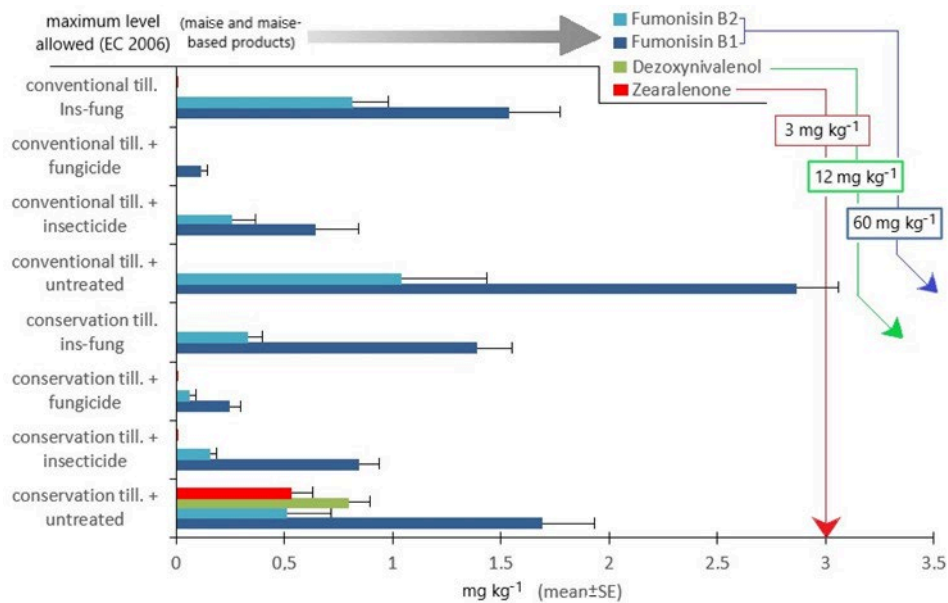


Figure 3. Treatment-dependent specific quantitative development of the tested toxins ($n=3$). Limits of detection (LOD) (mg kg^{-1}): FB1=0.03; FB2=0.01; DON=0.04; ZEA=0.005, and quantification (LOQ) (mg kg^{-1}): FB1=0.08; FB2=0.03; DON=0.12; ZEA=0.015

The study showed that insecticidal and fungicidal stand treatments reduce toxin contamination in plants independently of the tillage of the area. Two-way ANOVA demonstrated that field pesticide treatments have a statistically verifiable effect ($df=7$; $F=1.382$; $p \leq 0.001$) on the recorded values of measured fusarium toxins in maize and that there were significant differences in the quantitative values of measured toxins within treatments ($df=3$; $F=17.797$; $p \leq 0.001$). It was further confirmed that the interaction of toxin type and the combined effect of tillage and in-crop management interventions as treatments also had a statistically significant impact on the specific quantitative trend of toxin content ($df=21$; $F=1.835$; $p=0.033$). For the effects of different pesticide formulations on toxin contamination, the fungicide treatments applied in the middle of the growing season had the lowest toxin load. This was not far behind the toxin attenuation effect of the pure insecticidal treatments. Interestingly, the treatments with combined active substances did not achieve the expected toxin attenuation effect. However, the differences between treatments with different pesticide formulations were not statistically confirmed ($p > 0.05$).

Discussion

For many decades, the technology for producing grain maize has been based on an extensive approach. The cost conditions in the sector did not allow or justify additional treatments in the plant because of the phytosanitary status (Pimentel 2005). Climate extremes and the changes in pest spectrum and damage levels in maize are partly caused by them (Olesen & Bindu 2002); however, they have required a reassessment of this perception and a rethinking of the technological elements of maize soil and in-crop management (Difffenbaugh et al. 2008, Keszthelyi et al. 2009). The emergence of *D. v. virgifer*

in central Europe has generated the need to incorporate compulsory soil disinfestation of resown maize stands into the technology (Gray et al. 2009). *O. nubilalis*, the main mycotoxin intermediary with its chewing (Hutchison et al. 2010, Keszthelyi 2010), is making vegetation spraying an increasingly necessary element in sweet and seed production maize fields.

Our studies have shown that different tillage systems affect the greenness of maize stands and their photosynthetic activity recorded at later vegetation stages. Due to soil moisture retention, the conservative tillage system could provide water to the plants for a more extended period, as confirmed by our studies. This observation is in line with the previous findings of Jin et al. (2007). This is also supported by the higher mycotoxin concentrations observed in conventional tillage plots. Regardless of the absence or only moderate presence of plant residues in these fields, they provide overwintering and infection opportunities for important arthropod pests and toxin-producing fungi. Plants stressed and weakened by arid conditions were found to be more susceptible to attack by toxin-producing fungi (Dinolfo et al. 2022).

Among the pesticide treatments, cyantraniliprole, a well-absorbed and translocated non-approved spray in maize, effectively suppresses mastication pests (Xu et al. 2017). Its long-term effect resulted in much more moderate damage than untreated parcels. *O. nubilalis*, the most damaging pest at this time of year and the most conducive to mycotoxin-producing microfungi, had a damage rate well below 10% in several treatments (Labatte & Got 1991). This compound's ability gives it a significant advantage over pyrethroids, which are predominantly licensed in maize. This attribute also provides flexibility in the timing of control elements against *D. v. virgifer* (Labatte et al. 1996, Gagnon et al. 2019). Even if it can reduce the larval emergence in the case of a prolonged flight peak or if pesticide spraying is realized

earlier than scheduled. In addition, work by Gerbovits et al. (2024) has shown that this active ingredient uniquely enhances photosynthetic activity in plants.

The active substances prothioconazole and tebuconazole, known on the market as a toxin-controlling combination of active substances, have shown the expected toxin-scavenging effect in the vegetation cycle. Due to their application, the lowest toxin exposure levels were recorded in the treated parcels, coinciding with Ivanov and Harizanova's (2023) research results. An additional benefit of their application may be the enhanced photosynthetic activity of the treated plant (Blandino et al. 2012), which may also help avoid forced drying caused by arid conditions in the future.

Mycotoxin loads, an almost constant problem in European maize production today, certainly prompt a rethinking of the extensive practice of forage and grain maize cultivation. Any technological element that increases the photosynthetic activity of maize minimizes mycotoxin load, or reduces insect damage by killing microfungi or opening the way for their colonization is an essential contribution to the control of mycotoxicoses (Meissle et al. 2009). These desirabilities are met by using combinations of active substances timed to the peak of maize vegetation (tasselling), the future expansion of which is essential to improve the phytosanitary of maize.

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