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Impact of Diversified Chemical and Biostimulator Protection on Yield, Health Status, Mycotoxin Level, and Economic Profitability in Spring Wheat (*Triticum aestivum* L.) Cultivation

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Abstract: Biostimulators with chemical protection are a challenge in sustainable agriculture to obtain high yield, healthy, and pesticide-free wheat. The aim of this four-year spring wheat field experiment was to assess the effectivity of using herbicide, mixed fungicides protection, and a humic biostimulator. The following treatments were tested: biostimulator (S), sulfosulfuron (H), H + S, H + propiconazole + cyproconazole/spiroxamin + tebuconazole + triadimenol (H + F1 + F2), and H + F1 + F2 + S. Evaluations of wheat yield and fungal diseases (Septoria tritici blotch, eyespot, sharp eyespot, *Fusarium* spp.) were performed using visual and qPCR methods. Thirteen mycotoxins were analyzed by LC–MS/MS. Infestations of six weeds were examined visually. Temperatures and precipitation data of the vegetative seasons were monitored. Precipitation most affected the occurrence of leaf diseases despite the same chemical/biostimulator treatments (up to 48% Septoria tritici blotch severity for the S treatment). The highest mean yield was obtained for H + F1 + F2 + S (5.27 t ha^{-1}), while the lowest level of mycotoxins was obtained for H + F1 + F2 (221.68 µg kg^{-1}). For H + S, a greater reduction of mycotoxins was determined compared to the H treatment (27.18%), as well as a higher severity of eyespot (18%) and sharp eyespot (24%). In 2017–2020, the most effective reduction of weed infestation and *Fusarium* spp. DNA on ears was indicated for H + F1 + F2 (16 g and 0.88 pg g^{-1} DNA, respectively). The greatest saved production value (196.15€) was determined for H + F1 + F2 + S.

Keywords: biostimulator; fungal diseases; mycotoxins; pesticides

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1. Introduction

Cereal crops are the basic group of cultivated plants used worldwide for consumption, fodder, and industrial purposes. Cereals are rich in proteins, carbohydrates (including starch), fiber, phosphorus, zinc, silicon, fluorine, calcium, potassium, and B vitamins [1]. According to OECD/FAO [2], the estimated growth in wheat consumption by people will progress due to the growing human population. To increase the supply of wheat grain, crop protection methods contributing to higher yields and reduced occurrences of fungal diseases and mycotoxins need to be developed.

Wheat is susceptible to fungal pathogens which cause losses in yield and grain quality. Pathogenic fungi are responsible for leaf and stem diseases, contributing to severe yield and grain quality losses (e.g., *Septoria tritici* causes Septoria tritici blotch, *Tapesia yallundae* is responsible for eyespot, while *Rhizoctonia cerealis* causes sharp eyespot) [3]. Fungi belonging to *Fusarium* spp. are common microorganisms infecting cereals. In East-Central Europe, *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. poae*, and *F. oxysporum* occur most frequently [4]. Fusarium diseases in wheat cultivation include seedling blight, foot rot, and Fusarium

head blight (FHB), with the highest importance in agriculture. FHB is manifested by the bleaching of spikes and, due to the accumulation of mycotoxins in industrially used grains, it is the disease that requires the most attention in wheat cultivation [5]. According to the Köppen climate classification, Poland is a country in East-Central Europe with a humid continental climate and warm summer subtype. Compared to other countries of this region (e.g., Ukraine, Belarus, Estonia, Latvia, Lithuania, Slovakia, Romania, Hungary), Poland has similar fungal diseases and mycotoxins profiles of cereals [6]. It was noticed that high precipitation and temperature affect fungi development and mycotoxins secretion; however, less is known about the efficacy of sustainable crop protection in changing climate conditions.

Fusarium mycotoxins are commonly detected in pre-harvest cereals and post-harvest grains, food products, and animal feed, which contributes to food poisonings, cancers, and/or reproduction disorders during long-term intake. Fusarium mycotoxins commonly detected in the highest amounts in East-Central Europe include deoxynivalenol (DON; up to 153 μg kg^{-1}) [7], nivalenol (NIV; up to 150.8 μg kg^{-1}), zearalenol (ZON; up to 284 μg kg^{-1}), and HT-2 toxin (up to 40.8 μg kg^{-1}) [8]; however, their occurrence is specific to species and geographical location. Moreover, according to the European Food Safety Authority (EFSA) [9], most wheat grain samples collected in Europe have DON concentration between 15.5 μg kg^{-1} and 410 μg kg^{-1}, ZON between 3 μg kg^{-1} and 33 μg kg^{-1}, fumonisins between 37.4 μg kg^{-1} and 315 μg kg^{-1}, and NIV between 30 μg kg^{-1} and 430 μg kg^{-1}. According to the European Commission Regulation No. 1881/2006 [10], the maximum allowable concentrations of DON and NIV for unprocessed cereals in food processes are up to 1250 μg kg^{-1} and 750 μg kg^{-1}, respectively, for cereals intended for direct human consumption; 2000 μg kg^{-1} for the feed of calves and lambs; and 900 μg kg^{-1} for pigs' forage, while for 3-AcDON and 15-AcDON, up to 15–20% of DON can be achieved. Therefore, practical, diversified crop protection strategies are needed to reduce mycotoxins and provide safe grain intended for human and animal consumption. It was indicated that fludioxonil and difenoconazole are effective fungicides against the root rot of wheat, while carbendazim, thiophanate-methyl, and trifloxystrobin sufficiently combat FHB and in consequence limit the amount of mycotoxins in cereals [11,12].

Chemical protection is of major importance when it comes to the production of healthy crops (i.e., with the lowest possible level of mycotoxins). For the registration of plant protection products, the European Union was divided into three zones: the Baltic Sea, Mediterranean Sea, and Central Europe. Poland was classified in the Central zone together with Austria, Belgium, the Czech Republic, the Netherlands, Ireland, Luxembourg, Germany, Romania, Slovakia, Slovenia, Hungary, and Great Britain. Herbicides (triazolopyrimidines, sulfonoureas, and phenoxy acids) are basic pesticides used in East-Central Europe for crop protection and the reduction of weed infestation, which is the main factor limiting yield and grain quality and indirectly affecting disease severity [11]. Fungicides (triazoles, morpholines, strobilurins, and benzimidazoles) are often used as a support in herbicidal protection strategies, reducing the development of fungal diseases and, in consequence, limiting the level of mycotoxins and increasing yield and grain quality [12]. However, weed infestation is mainly investigated after single herbicidal treatments, while other pesticides, e.g., fungicides, can also affect their activity [13]. On the other hand, according to the "from field to fork" strategy of the European Commission, it is recommended to reduce the use of plant protection products in agriculture.

One of the methods of sustainable agriculture is the application of biostimulators combined with chemical protection. This solution is rare in practice but can be efficient in agriculture. Biostimulators are variable natural substances and microorganisms that alleviate the negative effects of abiotic stress, participate in the physiological and biochemical processes of plants, and stimulate their development and resistance to adverse growth conditions [14]. Biostimulators based on amino acids, sodium ortho nitrophenol, sodium para nitrophenol sodium 5-nitroguaiacolate, and plant extracts combined with MCPA, dicamba, florasulam, and 2,4-D can improve wheat yield and grain quality [15–19]. Moreover, bios-

timulators based on glutamic and organic acids, soluble carbohydrates, and microelements contributed to a higher yield, but their impact on fungal diseases and mycotoxins was not examined [20]. Biostimulators from brown algae can reduce mycotoxins level and *Fusarium* spp. occurrence; however, their effect on yield and leaf diseases was not investigated [21]. Humic and fulvic acids, which are components of humic biostimulators, have a positive effect on the development of lateral roots, aerial parts growth stimulation, or mitigation of the effects of water shortages and soil salinity [16]. However, their impact on fungal diseases severity and the reduction of mycotoxins level was poorly investigated. Moreover, there are limited studies concerning the effect of biostimulators combined with pesticides on health status and weed infestation in agricultural plants [17] with the practical aspect of economic efficiency.

The new insights of this study include a comprehensive assessment of the impact of various levels of chemical protection combined with a biostimulator or the exclusive use of a biostimulator as well as climatic conditions of East-Central Europe on the health status of wheat. The collateral goals were as follows: (1) evaluation of the impact of herbicide, herbicide with fungicides, and herbicide with fungicides and biostimulator on wheat yield and ergosterol content; (2) examination of fungal diseases severity and mycotoxins concentration for different crop protection strategies; (3) study of the mutual relationship between climatic conditions and quantitative and qualitative wheat grain parameters according to the diversified level of chemical/biostimulator protection; (4) and the determination of the economic profitability of different protection strategies in agricultural practice.

Taking into account the fact that the zonal registration of plant protection products in the European Union coincides with the climate zone of East-Central Europe, the results of our research can also be applied to other countries in the region.

2. Materials and Methods

2.1. Field Experiment and Meteorological Data

Spring wheat (*Triticum aestivum* L.) of the Mandaryna variety was cultivated in the experimental plots (4 m × 5 m; total area 640 m^2) over four years in northeastern Poland (53°11′43.6″ N 23°01′02.7″ E; 165 m AMSL) with a humid continental climate and warm summer subtype. After wheat harvest in each year, lupine was grown on the field as an aftercrop. The climatic conditions of this area are similar to other countries of East-Central Europe [6]. Certified seeds were sown on 4 April 2017, 6 April 2018, 3 April 2019, and 7 April 2020. Seedlings (except controls) were sprayed with commercial pesticides including herbicide sulfosulfuron (H, active substance; a.s.: 75%), applied at BBCH 31 and 26.5 g ha^{-1}; fungicides (in recommended rates) F1 (propiconazole, a.s.: 22.4% + cyproconazole, a.s.: 7.16%), applied at BBCH 32 and 200 mL ha^{-1} and F2 (spiroxamine, a.s.: 25.25%, tebuconazole, a.s.: 16.87%, triadimenol, a.s.: 4.34%), applied at BBCH 65 and 600 mL ha^{-1}; and a humic biostimulator improving plant growth (S, humic and fulvic acids >95%; C, N, P_2O_5, K_2O, Cu, Zn, Mn, Ni <5%; applied at BBCH 33, 47, 72; 250 mL ha^{-1}) (Florahumus, Sieniawa Lubuska, Poland). Commercial herbicide was purchased from Monsanto (Creve Coeur, MO, USA), commercial fungicide F1 was obtained from Syngenta (Basel, Switzerland), and commercial fungicide F2 was purchased from Bayer (Leverkusen, Germany). Treatments were carried out with a compressed air backpack sprayer with 4 nozzles (XR Tee Jet; 110 02 XR, 145–225 µm, and 110 03 XR, 226–325 µm) at a liquid flow rate of 200 L ha^{-1}. Details of the treatments are listed in Table 1. The experiment consisted of 5 combinations and 4 repetitions each. Nitrogen (N)/phosphorous (P)/potassium (K) fertilization in each year was carried out as follows: 50 kg N ha^{-1}, 60 kg K ha^{-1}, and 21 kg P ha^{-1}. The physical and chemical soil parameters indicated a pH of 7.3 and a microelements content of 1.64 mg kg^{-1} K_2O, 1.72 mg kg^{-1} P_2O_5, and 0.93 mg kg^{-1} Mg. Grains were harvested from each plot at the BBCH 89 stage on 27 July 2017 (1st year), 30 July 2018 (2nd year), 25 July 2019 (3rd year), and 22 July 2020 (4th year). Next, grains were separated from husks and subjected to further analysis.

Table 1. Details of the treatments carried out in this study.

No.	Treatment	Active Ingredient	Active Ingredient Dose (g, mL ha^{-1})	Wheat BBCH Stage for Application
1	C (control)	-	-	-
2	S	Humic and fulvic acids	250 mL ha^{-1}	33, 47, 72
3	H	Sulfosulfuron	26.5 g ha^{-1}	31
4	H + S	Sulfosulfuron/humic and fulvic acids	26.5 g ha^{-1}/250 mL ha^{-1}	31/33, 47, 72
5	H + F1 + F2	Sulfosulfuron/propiconazole + cyproconazole/spiroxamin + tebuconazole + triadimenol	26.5 g ha^{-1}/200 mL ha^{-1}/ 600 mL ha^{-1}	31/32/65
6	H + F1 + F2 + S	Sulfosulfuron/propiconazole + cyproconazole/spiroxamin + tebuconazole + triadimenol/humic and fulvic acids	26.5 g ha^{-1}/200 mL ha^{-1}/ 600 mL ha^{-1}/250 mL ha^{-1}	31/32/65/33, 47, 72

Mean precipitation in the vegetative season was 295 mm in 2017 with a temperature of 13.25 °C, 204 mm and 16.41 °C in 2018, 183 mm and 14.9 °C in 2019, and 160 mm and 13.7 °C in 2020. The temperature and precipitation data of the vegetative seasons were obtained from the meteorological station located at the experimental plots (53°11′43.6″ N 23°01′02.7″ E).

2.2. Evaluation of Wheat Yield and Fungal Diseases

Wheat yield was assessed by harvesting all ears from 20 m^2 with a plot harvester; weighting and weight from 20 m^2 was extrapolated to 1 ha. Ergosterol content was assessed using an Infratec 1241 device (Foss, Hilleroed, Denmark), based on NIR (near-infrared radiation). Fungal diseases (Septoria tritici blotch, eyespot, sharp eyespot) were evaluated visually at the milk-dough growth stage (BBCH 79) in the stem base, flag, and second leaf on 25 randomly collected plants from each plot, according to the EPPO (European and Mediterranean Plant Protection Organization) scale. Disease severity was determined as low at <20%, moderate at 20–40%, and high at >40%.

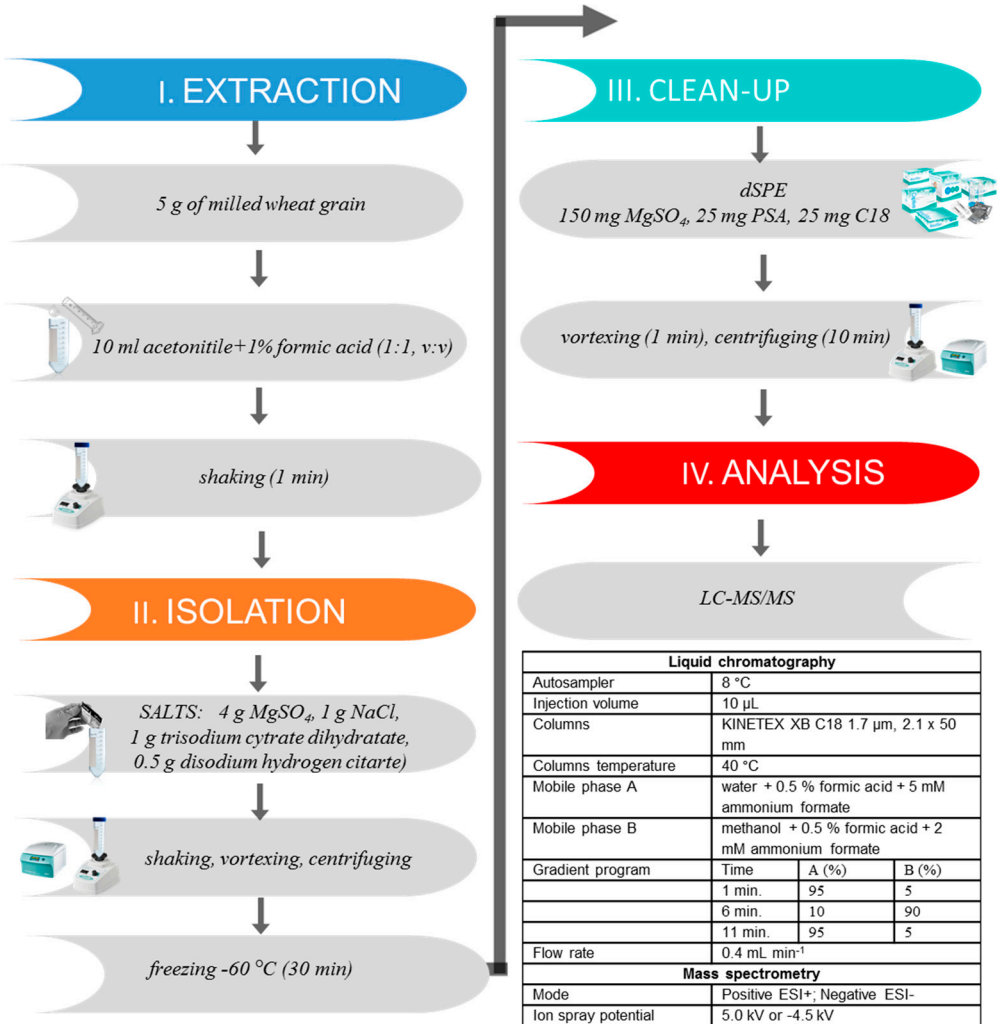
2.3. Quantitative Determination of *Fusarium* spp.

F. culmorum, *F. avenaceum*, *F. graminearum*, *F. poae*, and *F. oxysporum* reference strains were grown for 5 days in 23 °C on potato dextrose agar (PDA). Next, 100 mg of mycelium was scraped from the solid medium and DNA isolation was performed according to the modified for filamentous fungi CTAB method with a NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer.

The aggregate sample of grain (20 g) originating from each experimental plot was ground in the mortar and 40 mg of flour was taken for DNA isolation. DNA was extracted using a modified CTAB method with a NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). The concentration and purity of the isolated DNA were measured in a NanoPhotometer P300 (Implen, Munich, Germany). Next, DNA wheat samples were diluted to the working concentration 100 ng μL^{-1} and kept at −20 °C for further analysis. Real-time PCR with serial dilutions of *Fusarium* spp. DNA as positive controls was performed to assess accuracy and specificity in amplifying sequences of the *EF1-alpha* gene with following primers: *F. culmorum* (5′-GTAATTTTTCTGGTGGGGCT-3′ and 5′-AACTGATTGACACGTGATGG-3′), *F. avenaceum* (5′-ATTCATTACCCCGCTCAAGT-3′ and 5′-TGTGGTAAGGTTTTGTGGGA-3′), *F. graminearum* (5′-TATCATTCGAATCGCCCTCAC-3′ and 5′-GACAGGTGGTTAGTGACTGGT-3′), *F. poae* (5′-GCTAACATGCTTGACAGACC-3′ and 5′-ATGGATCGAGGGAAAGTAGG-3′), and *F. oxysporum* (5′-CATACTGACATCGTTT CACAG-3′ and 5′-TAGCGGGTACGTTTCGAGT-3′). Real-time PCR for fungal and wheat samples was performed according to a previously described protocol [4]. Obtained *Fusarium* spp. concentrations were calculated as pg fungal DNA g^{-1} dry mass (dm).

2.4. Determination of Mycotoxins

The mycotoxins deoxynivalenol (DON) and its acetylated forms (3-AcDON, 15-AcDON), nivalenol (NIV), zearalenone (ZON), diacetoxyscirpenol (DAS), fusarenon X (FusX), T-2, HT-2, fumonisins (FUM B1, FUM B2, FUM B3), and neosolaniol (NEO) were obtained from LGC (Wasel, Germany). Individual stock solutions were prepared in acetonitrile/water (1:1, *v*/*v*) at a concentration of 1 mg mL^{-1} and were used to obtain a standard mixture at the concentrations of 0.1–1000 µg mL^{-1}. The standard mixture was stored at −18 °C. Mycotoxins were extracted using the QuEChERS method and analyzed via LC–MS/MS based on previously described protocols [8,22] (Figure 1), followed by the validation according to the Document No. SANTE/11813/2017 [23]. The details of the full analytical procedure and validation data are given in Appendix A (Tables A1–A3). A chromatogram of representative mycotoxins is shown in Figure S1.



Liquid chromatography			
Autosampler	8 °C		
Injection volume	10 µL		
Columns	KINETEX XB C18 1.7 µm, 2.1 x 50 mm		
Columns temperature	40 °C		
Mobile phase A	water + 0.5 % formic acid + 5 mM ammonium formate		
Mobile phase B	methanol + 0.5 % formic acid + 2 mM ammonium formate		
Gradient program	Time	A (%)	B (%)
	1 min.	95	5
	6 min.	10	90
	11 min.	95	5
Flow rate	0.4 mL min^{-1}		
Mass spectrometry			
Mode	Positive ESI+; Negative ESI-		
Ion spray potential	5.0 kV or -4.5 kV		
Temperature of turbo heaters	400 °C		
Scan type	Multiple reaction monitoring		
Quantification	Precursor ion and 2 product ions		

Figure 1. Scheme of wheat grain sample preparation, extraction, and analysis of mycotoxins using LC–MS/MS.

2.5. Weed Infestation

To assess weed infestation, all plants excluding wheat were uprooted from 1 m^2 of control and all treatments. The plant material was collected in the BBCH 54 stage of wheat (heading). Weeds were cleaned from the soil particles. On the day of harvesting, the

number and mass of the following most common species were determined: shepherd's purse (*Capsella bursa-pastoris*), white goosefoot (*Chenopodium album*), common knotgrass (*Polygonum aviculare*), red pimpernel (*Anagallis arvensis*), yellow foxtail (*Setaria glauca*), black bindweed (*Fallopia convolvulus*), and wall speedwell (*Veronica arvensis*).

2.6. Economic Optimum Rate

To assess the economic profitability of each protection strategy, the following calculations were performed:

$$E = Spv/Ct \quad (1)$$

$$Spv = Ys \times C \quad (2)$$

$$E = (Ys \times C)/Ct \quad (3)$$

$$Ym = Ct/C \quad (4)$$

where E is economic the efficiency (>1), Spv is the saved production value (€), Ct is the total cost of treatment (cost of plant protection products, biostimulator, average cost of fuel per hectare), Ys is the yield saved from a particular strategy compared to the control (t ha^{-1}), C is the average cost of wheat at harvest for each study year (t ha^{-1}), and Ym is the minimum yield saved in a particular strategy compared to the control, justifying the profitability of the treatment. An economic efficiency above 1 indicates that the strategy is profitable in agricultural practice. The above equations were calculated for average yield and costs from the four-year study. The average costs of treatment per 1 ha in relation to the prices in Poland were as follows: biostimulator (6.16 €), herbicide (24.78 €), herbicide + biostimulator (30.94 €), herbicide + fungicide F1 + fungicide F2 (63.32 €), and herbicide + fungicide F1 + fungicide F2 + biostimulator (69.48 €). The average price of 1 t of wheat was 163.45 €.

2.7. Statistical Analysis

Analyses were conducted using one-way ANOVA and a post hoc Fisher's test (Table S1). A principle component analysis (PCA) between variables was performed. A resulting correlation matrix was visualized as a heatmap. Statistical significance was established as $p \leq 0.05$. For the examined traits, Pearson's correlation (r) was carried out for $p \leq 0.05$. All data were elaborated in STATISTICA 12 software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Quality Parameters of Wheat Grain under Diverse Chemical/Biostimulator Treatments

Despite frequent single herbicidal protection, the highest yield in the years 2017–2020 was determined for the sulfonylurea herbicide with a biostimulator (H + S; 6.5 t ha^{-1}), herbicide combined with morpholine/triazole fungicides (H + F1 + F2; 6.3 t ha^{-1}), and the herbicidal and fungicidal treatment combined with a humic biostimulator (H + F1 + F2 + S; 6.1 t ha^{-1}) (Table 2). In the four-year period, an average yield ranged from 3.43 t ha^{-1} in 2017 to 6.5 t ha^{-1} in 2018. Low yields for all combinations in 2017 are connected with high total precipitations, and they resulted in greater fungal diseases incidence. However, single biostimulator application caused a lower yield (4.8 t ha^{-1}) compared to that of the control (5.3 t ha^{-1}).

Table 2. Quantitative and qualitative parameters of spring wheat grain under diverse chemical/biostimulator treatments (disease severity: low <20%, moderate 20–40%, and high >40%). The same letter in the particular year of the study indicates that the value is not significantly different ($p \geq 0.05$).

No.	Treatment [1]	Year	Yield (t ha^{-1})	Ergosterol (mg kg^{-1})	Flag Leaf	Stem Basis		*Fusarium* spp. (pg g^{-1})	Mycotoxins (µg kg^{-1})
					S [2] (%)	E [3] (%)	SE [4] (%)		
1	C	1	3.43 a	9 bcd	11 a	19 a	15 a	15.27 a	29.1 a
		2	5.3 b	9.2 a	6 a	27 a	16.5 a	5.96 a	983 a
		3	3.7 ab	8 a	-	6.8 a	1.1 a	2.44 a	823.9 a
		4	3.9 a	9.7 a	-	1.8 a	1.2 a	7.46 a	912.5 a
2	S	1	3.9 ab	7.8 abc	48 b	17 a	17 a	15.52 a	51.9 b
		2	4.8 a	9.2 a	6 a	6.2 b	9.6 b	1.76 b	383.7 b
		3	3.4 a	7.1 a	-	3 a	2.1 a	1.90 b	895.3 a
		4	3.8 a	10.3 b	-	1.6 a	0.4 b	17.35 b	988.8 a
3	H	1	4.5 bc	9.9 d	19 a	21 a	12 a	13.35 a	52.7 b
		2	5.4 b	9.2 a	5.8 a	24.1 a	13.1 c	2.29 b	641.1 c
		3	4.3 bcd	7.7 a	-	6.2 a	0.7 a	1.97 b	667 b
		4	4.4 b	9.3 a	-	3.6 b	0.5 b	2.27 c	684.1 b
4	H + S	1	4.09 ab	9.3 cd	43 b	28 a	24 a	10.52 b	75.2 c
		2	6.5 e	9.1 a	5.5 a	25.1 a	12.3 bc	8.38 d	466.8 d
		3	4.4 cd	7.4 a	-	3.1 a	1.3 a	2.01 bc	589.8 c
		4	4.3 b	9.7 a	-	2.6 ab	0.7 ab	1.39 d	671.5 b
5	H + F1 + F2	1	4.82 bc	7 ab	47 b	17 a	17 a	7.47 c	13.5 d
		2	6.3 de	7.9 b	3.5 b	18 c	14 a	0.88 e	278.5 e
		3	4.6 cd	7.4 a	-	5 a	0.7 a	1.74 c	314.1 e
		4	4.7 c	8.3 c	-	2.2 a	0.5 b	1.68 d	280.6 d
6	H + F1 + F2 + S	1	5.16 c	6.9 a	42 b	22 a	21 a	8.34 c	31.7 a
		2	6.1 cd	8.8 a	3.1 b	22.7 c	22.8 d	1.86 b	230.4 e
		3	4.9 d	6.8 a	-	4.5 a	0.6 a	1.56 c	390 e
		4	4.9 c	8.4 c	-	1.7 a	0.1 b	2.26 e	314.1 de

[1] Treatment: C—control; S—biostimulator; H—herbicide; F1—fungicide 1; F2—fungicide 2. [2] Septoria tritici blotch. [3] Eyespot. [4] Sharp eyespot.

Septoria tritici blotch was observed on leaves, while eyespot and sharp eyespot were noticed on the stem. However, according to EPPO recommendations, the severity of Septoria tritici blotch was determined as high only in 2017 (>40%) for selected combinations (Table 2). Additionally, in 2018, eyespot was noticed as moderate (20–40%) for the control and sulfosulfuron (H), the sulfosulfuron with biostimulator (H + S), and the herbicide combined with fungicides and biostimulator (H + F1 + F2 + S). A moderate severity of sharp eyespot was determined in 2017 and 2018 for selected combinations. In 2019 and 2020, the severity of all examined fungal diseases was determined as low (<20%). Other diseases and pests were not detected.

Ergosterol is a marker of fungal infection in plants. The most effective reduction of its concentration was determined in fungicidal treatments (Table 2) ($p < 0.05$). Additionally, ergosterol content was also lower following the application of a biostimulator in 2017 and 2019. The total concentration of ergosterol may be related to the occurrence of other fungi, which were not assessed in this study due to their low incidence.

3.2. Evaluation of *Fusarium* spp. and Their Metabolites Concentration in Wheat Grain

The occurrence of *Fusarium* spp. may be related to climatic conditions. Our results show that the total *Fusarium* spp. concentration in the four years of the study was variable. The highest content of *Fusarium* spp. in most combinations (up to 15.63 pg DNA g^{-1} dm) was determined in 2017 (Figure 2) and was related to the highest precipitation and a lower temperature in this year of the study. Species composition was also diverse in individual years of the study. *F. culmorum* was the predominant pathogen in 2017 (7.87 pg g^{-1} for the single biostimulator treatment), *F. oxysporum* in 2018 (4.84 pg g^{-1} for the control), and *F. graminearum* in 2019 (1.27 pg g^{-1} for the control). *F. poae* occurred in the lowest concentration, especially in 2018–2019. Moreover, in 2020, the predominant species in wheat from the control and biostimulator treatment was *F. graminearum* (15.76 pg g^{-1}).

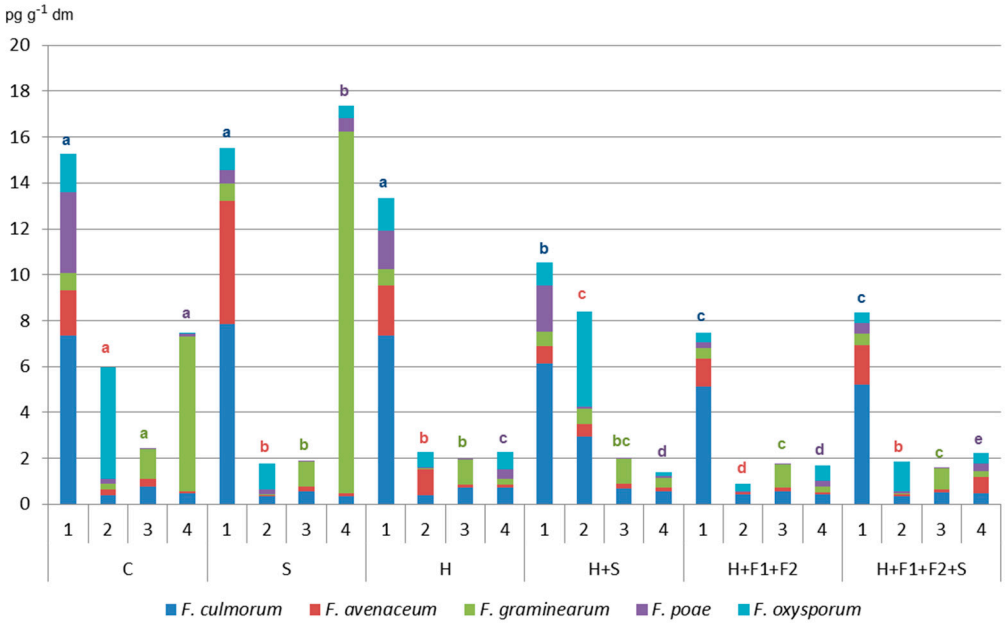


Figure 2. Concentration of *Fusarium* spp. in wheat grain in four years of the study (1—2017; 2—2018; 3—2019; 4—2020). C—Control; S—biostimulator; H—herbicide; F1—fungicide 1; F2—fungicide 2. Bars marked with the same letter do not differ significantly ($p \geq 0.05$) in the particular year of the study.

The sulfonylurea herbicide combined with morpholine and triazoles fungicides (H + F1 + F2) contributed to the most effective reduction of *Fusarium* spp. concentration from 7.47 pg g^{-1} in 2017 to 0.88 pg g^{-1} in 2018 (decrease of 61.57% in 2018 and 44.04% in 2017, compared to the single herbicide treatment) (Figure 2).

The mycotoxins profile was diversified in particular years of the study. From the 13 examined mycotoxins, 8 were detected in 2017 (3-AcDON, 15-AcDON, DON, NIV, ZON, HT-2, FUM B1, FUM B2), and 5 in 2018–2020 (3-AcDON, 15-AcDON, DON, NIV, ZON) (Table 3, Figure 3). Figure S1 shows the example of a chromatogram of mycotoxin standards and mycotoxins detected under the herbicide treatment of wheat in 2020. The total level of mycotoxins was the highest in 2020 (988.8 µg kg^{-1} for the biostimulator treatment) and the lowest in 2017 (13.5 µg kg^{-1} for the sulfonylurea herbicide combined with morpholine and triazole fungicides). Generally, treatments with morpholine and triazole fungicides were the most effective in reducing the total amount of mycotoxins (13.5 µg kg^{-1}, 278.5 µg kg^{-1}, 314.1 µg kg^{-1}, 280.6 µg kg^{-1} in 2017–2020, respectively; reduction of 53%, 72%, 62%, 69%, respectively). In 2020, the lowest total precipitation and *Fusarium* spp. concentration, but also the greatest level of mycotoxins, were observed. Interestingly, despite the lack of fungicides application, the sulfosulfuron treatment combined with a humic biostimulator (H + S) reduced mycotoxins content to 466.8 µg kg^{-1} in 2018 (27%), 589.8 µg kg^{-1} in 2019 (12%), and 671.5 µg kg^{-1} in 2020 (2%), compared to exclusive herbicide application (Figure 3).

Table 3. Mycotoxins concentration in diverse strategies of crop protection in four years of the study (1—2017; 2—2018; 3—2019; 4—2020). The same letter in the particular year of the study indicates that the value is not significantly different ($p \geq 0.05$).

			Mycotoxins (µg kg^{-1})								
No.	**Treatment** [1]	**Year**	**DON**	**3-AcDON**	**15-AcDON**	**NIV**	**ZON**	**FUM B1**	**FUM B2**	**HT-2**	**Total**
1	C	1	15.7 a	0.8 a	0	0	8.5 a	1.7 ac	0	2.4 a	29.1 a
		2	229 d	24 e	83.1 d	607 e	39.9 f	0	0	0	983 a
		3	234 a	24.3 a	67.9 a	450 a	47.7 a	0	0	0	823.9 a
		4	243 a	31.6 a	72.4 a	512 a	53.5 a	0	0	0	912.5 a
2	S	1	35.5 b	0.5 a	0	0	8.4 a	4.7 b	0	2.8 a	51.9 b
		2	55.8 b	7.9 c	43.2 bc	256 bc	20.8 d	0	0	0	383.7 b
		3	246 a	23.7 a	64.3 a	532 b	29.3 b	0	0	0	895.3 a
		4	237 a	29.8 a	63.2 a	603 b	55.8 a	0	0	0	988.8 a
3	H	1	39.6 b	1.3 a	0	0	8.3 a	1.9 a	0	1.6 b	52.7 b
		2	131 c	13.5 d	46.1 c	425 d	25.5 e	0	0	0	641.1 c
		3	190 b	24 a	53.5 ab	358 c	41.5 a	0	0	0	667 b
		4	173 b	16.6 b	40.8 b	411 c	42.7 b	0	0	0	684.1 b
4	H + S	1	65.6 c	0.8 a	0	0	7.6 a	1.2 a	0	0 c	75.2 c
		2	64.8 b	5.4 b	35.5 b	345 cd	16.1 c	0	0	0	466.8 d
		3	163 bc	14.6 b	44.7 bc	339 c	28.5 b	0	0	0	589.8 c
		4	144 c	13.9 b	44.6 b	425 c	44 b	0	0	0	671.5 b
5	H + F1 + F2	1	2.7 d	1.1 a	0	0	7.4 a	2.0 c	0	0.3 d	13.5 d
		2	28.6 a	4.5 b	33.3 b	205 ab	7.1 b	0	0	0	278.5 e
		3	88.2 de	3.5 c	26.8 d	186 e	9.6 f	0	0	0	314.1 e
		4	80.1 de	3 c	17.8 d	171 e	8.7 d	0	0	0	280.6 d
6	H + F1 + F2 + S	1	18.3 a	0.5 a	0	0	8.1 a	2.3 c	0.6 a	1.9 b	31.7 a
		2	14.3 a	1.9 a	14.8 a	195 ab	4.4 ab	0	0	0	230.4 e
		3	67.3 d	5.8 c	39.2 ce	261 d	16.7 ce	0	0	0	390 e
		4	72.1 d	3.3 c	28.9 c	199 ef	10.8 d	0	0	0	314.1 de

[1] Treatment: C—control; S—biostimulator; H—herbicide; F1—fungicide 1; F2—fungicide 2.

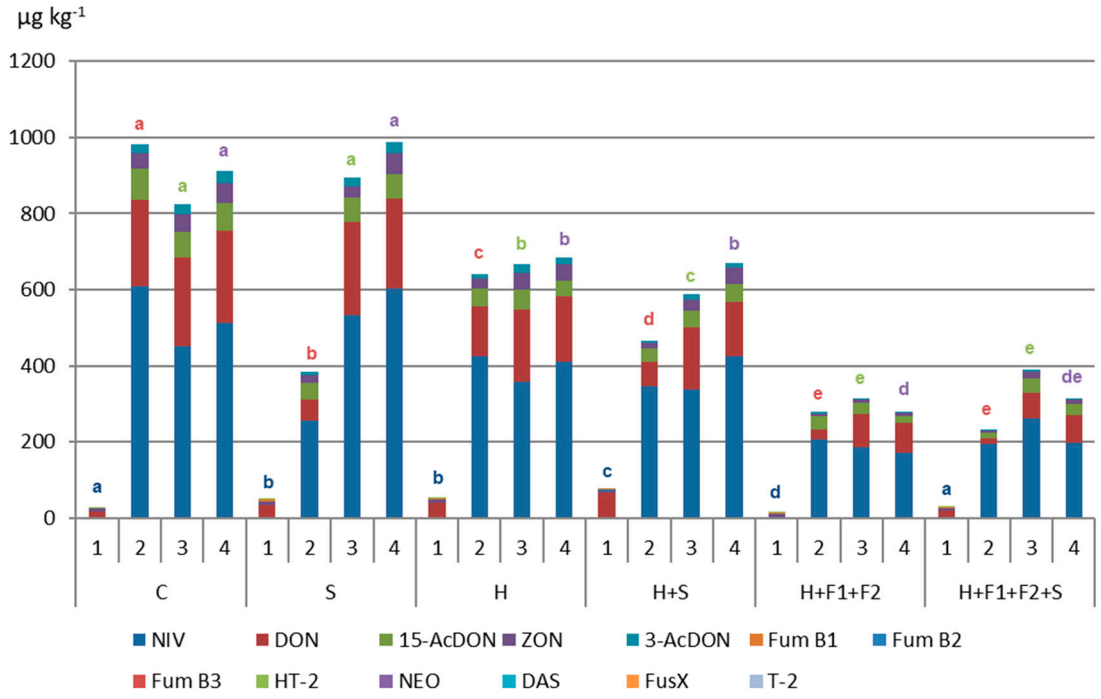


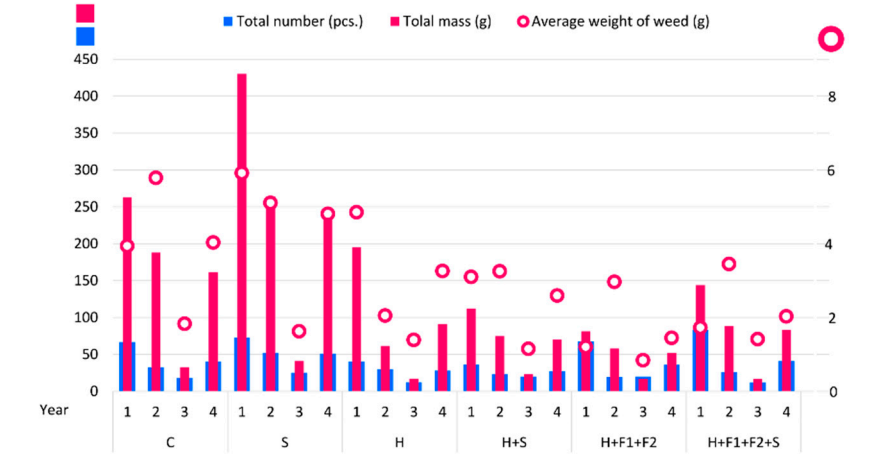
Figure 3. Mycotoxins concentration in wheat grain in four years of the study (1—2017; 2—2018; 3—2019; 4—2020). C—Control; S—biostimulator; H—herbicide; F1—fungicide 1; F2—fungicide 2. Bars marked with the same letter do not differ significantly ($p \geq 0.05$) in the particular year of the study.

Deoxynivalenol (DON) was predominant only in 2017, while NIV was determined to have the highest concentration in 2018–2020 (Table 3). Additionally, HT-2, FUM B1, and FUM B2 were noticed only in 2017.

3.3. Weed Infestation

Weed infestation is a main factor affecting crop quantitative and qualitative parameters. Thus, exclusive fungicidal treatments are not performed in agricultural practice. In the four-year period of the research, seven common weed species were noticed: *Capsella bursa-pastoris*, *Chenopodium album*, *Polygonum aviculare*, *Anagalis arvensis*, *Setaria glauca*, *Fallopia convolvulus*, and *Veronica arvensis*. The number of weeds was the highest in 2017 (up to 84 pieces per 1 m^2) and was caused by the greatest precipitation (293 mm).

Biostimulator application resulted in the greatest biomass of weeds compared to the control (430 g) (Figure 4). Herbicide combined with fungicides and enriched by a humic biostimulator caused a higher number and biomass of weeds compared to the treatment without biostimulator addition (84 pieces per 1 m^2 and 145 g, a 44% increase). Interestingly, the most efficient reduction of weed infestation in 2017–2020 was noticed for the sulfonylurea treatment combined with morpholine and triazole fungicides (up to 12 pieces per 1 m^2 and 16 g in 2019). However, weed biomass is a more effective method for weed infestation evaluation.



Year		1				2				3				4			
Month	Decade	Precipitation (mm)		Temperature (°C)		Precipitation (mm)		Temperature (°C)		Precipitation (mm)		Temperature (°C)		Precipitation (mm)		Temperature (°C)	
April	I	50%	5.7	168%	9.7	250%	28.5	160%	9.3	40%	4.6	106%	6.2	0%	0.0	110%	6.4
	II	165%	20.5	52%	3.9	12%	1.5	180%	13.3	0%	0.0	76%	5.6	15%	1.8	69%	5.1
	III	300%	34.8	55%	5.6	59%	6.8	134%	13.5	22%	2.6	139%	14.1	22%	2.6	98%	9.9
May	I	249%	52.3	84%	9.3	0%	0.0	147%	16.3	7%	1.4	76%	8.4	44%	9.2	97%	10.8
	II	0%	0.0	99%	13.1	42%	9.7	117%	15.4	170%	39.5	107%	14.1	120%	27.9	71%	9.4
	III	25%	6.0	101%	16.0	52%	12.3	115%	18.1	69%	16.5	98%	15.5	82%	19.4	78%	12.3
June	I	60%	10.5	91%	15.0	24%	4.3	106%	17.5	24%	4.3	122%	20.1	161%	28.4	99%	16.4
	II	261%	66.8	103%	16.9	14%	3.5	119%	19.5	27%	7.0	137%	22.5	118%	30.3	121%	19.9
	III	106%	27.8	101%	17.3	56%	14.7	99%	17.1	27%	7.2	117%	20.1	48%	12.7	119%	20.5
July	I	39%	18.9	87%	15.9	39%	18.9	92%	16.9	66%	31.7	88%	16.1	41%	19.9	103%	18.8
	II	52%	18.7	93%	17.2	247%	88.8	110%	20.5	68%	24.4	88%	16.4	21%	7.4	95%	17.6
	III	136%	31.2	100%	19.2	83%	19.1	120%	23.0	191%	43.7	104%	20.1	4%	1.0	93%	17.9
Total precipitation/ Average temperature		105%	293.2	94%	13.3	74%	208.1	118%	16,7	65%	182.9	106%	14.9	57%	160.6	97%	13.7

Figure 4. Total weed infestation in wheat tillage and climatic conditions: temperatures (°C) and precipitation (mm) of northeastern Poland (1—2017; 2—2018; 3—2019; 4—2020). Percentage values are related to the mean from the last decade (values below 100% indicate temperature/precipitation decrease relative to the last 10 years). C—Control; S—biostimulator; H—herbicide; F1—fungicide 1; F2—fungicide 2.

3.4. Economic Optimum Rates of Different Protection Strategies

Economic calculations indicated that all protection strategies were profitable in agricultural practice (E > 1), except for the exclusive biostimulator treatment (E = −1.55) (Figure 5). However, the greatest economic efficiency was noticed in the case of the exclusive sulfosulfuron treatment (E = 3.36) despite the lowest saved production value (98.07 € for 0.6 t ha^{-1}).

Moreover, the highest yield saved (1.2 t ha^{-1}) and the greatest saved production value (196.15 €) due to the protection strategies were determined in the herbicidal and fungicidal treatment combined with a biostimulator (H + F1 + F2 + S). Furthermore, taking under consideration the total costs of the treatment and the greatest minimum yield, the lowest economic efficiency among the chemical trials was noticed for sulfonylurea, morpholine, and triazoles (H + F1 + F2) (E = 2.41) (Figure 5).

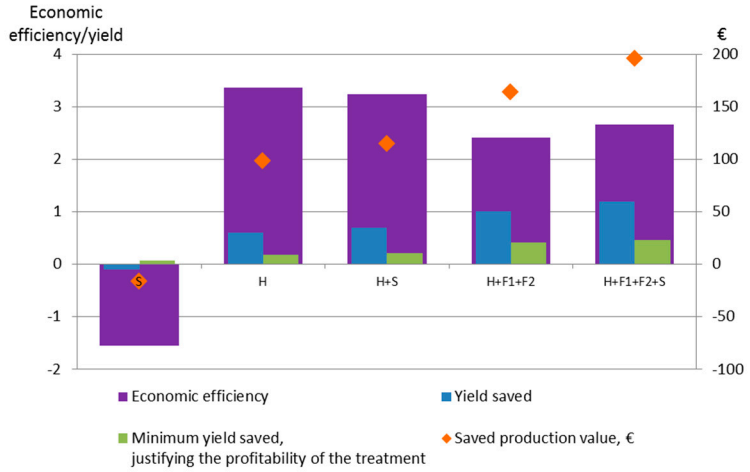
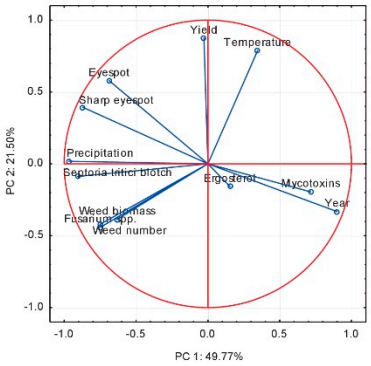


Figure 5. Economic efficiency, yield saved, minimum yield justifying the profitability of the treatment, and saved production value between diverse protection strategies. Figure shows average values from four seasons.

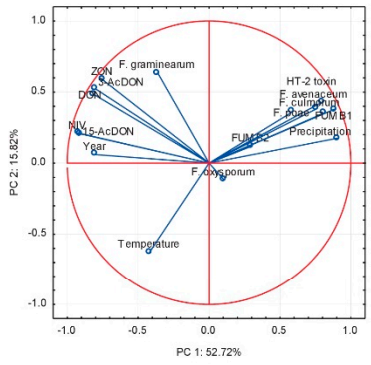
3.5. Statistical Analysis

In order to understand the significance of the results obtained, statistical analysis was performed. Figure 6a,b shows the PCA analysis indicating the influence of the type of chemical treatment, temperature, and precipitation on yield, ergosterol content, Septoria tritici blotch, eyespot, sharp eyespot, *Fusarium* spp., mycotoxins concentration in grain, weed number, and biomass in wheat cultivation in 2017–2020. The principal component analysis explained 71.27% of the total variability among all the examined parameters and 68.54% of the variability between the mycotoxins, *Fusarium* spp., and climatic conditions. During the four-year study, in 2017, the highest precipitation, disease severity, and *Fusarium* spp. concentration along with the lowest temperature and level of mycotoxins were observed. The heatmap of the examined parameters based on Pearson's correlation coefficients indicated a positive correlation between precipitation and Septoria tritici blotch, eyespot, and sharp eyespot (r = 0.9, r = 0.64, r = 0.83, respectively) (Figure 6c). Additionally, a correlation between eyespot and sharp eyespot (r = 0.86) was determined. Moreover, a positive correlation between precipitation and *Fusarium* spp. (r = 0.69) was indicated and a negative correlation was observed between precipitation and mycotoxins (r = −0.73). Weed number and biomass were positively correlated with precipitation (r = 0.64; r = 0.49, respectively), Septoria tritici blotch (r = 0.68; r = 0.47, respectively), sharp eyespot (r = 0.49; r = 0.42, respectively), and *Fusarium* spp. (r = 0.69; r = 0.74, respectively), and negatively correlated with temperature (r = −0.54; r = −0.29, respectively) and mycotoxins concentration (r = −0.43; r = 0.24, respectively). Ergosterol did not significantly correlate with any of the examined parameters, while wheat yield was positively correlated with temperature (r = 0.62) and eyespot (r = 0.51), and negatively correlated with *Fusarium* spp. (r = −0.37). The four-year study indicated that 3-AcDON, 15-AcDON, DON, NIV, and ZON were negatively correlated with precipitation (up to r = −0.76), while HT-2 and FUM B1 were positively correlated (r = 0.75, r = 0.82, respectively) (Figure 6c). Our study indicated negative correlations between *F. culmorum*, *F. avenaceum*, and 3-AcDON, 15-AcDON, DON, NIV, and ZON (up to r = −0.71) and positive correlations between *F. culmorum*, *F. avenaceum*,

and HT-2 and FUM B1 (up to r = 0.88). In the four-year study, *F. graminearum* was positively correlated with 3-AcDON, 15-AcDON, DON, NIV, and ZON.



(a)



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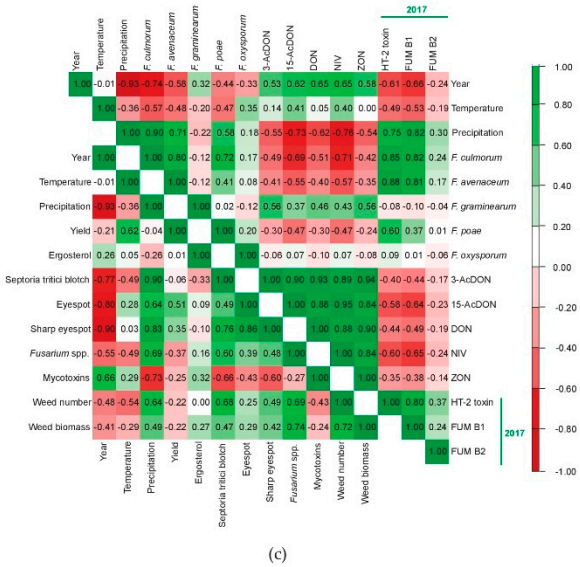

(c)

Figure 6. (**a**) Principal component analysis of the chemical and biostimulator treatment impact on the examined wheat grain parameters in 2017–2020. (**b**) Detailed principal component analysis of the chemical and biostimulator treatment impact on the particular *Fusarium* spp. and mycotoxins occurrence in 2017–2020. (**c**) Heatmap based on Pearson's correlation coefficients. Mutual correlation between examined parameters in wheat grain in 2017–2020.

4. Discussion

Despite the same pesticide/biostimulator protection during the four years of the study, precipitation and temperature had the most influence on the examined parameters and

uncertainties between them. Moreover, biostimulator treatments are more sufficient in warmer years and higher air humidity decreases their efficacy.

Our results indicated an improvement of wheat yield after the application of a biostimulator based on humic acids. It was also determined that other biostimulators, e.g., with plant hormones, resulted in a higher yield [21], but algae extract and nitrophenol biostimulators have no effect or slightly reduce wheat yield if combined with pesticides [24]. Apart from sulfosulfuron in wheat, an improvement in yield was also indicated for pyrazosulfuron singly applied [25]. However, yield increase after herbicide application is not obvious. A lower wheat yield was determined under isoproturon protection [24].

The use of fungicides prior to the onset of disease symptoms was indicated to be the most effective strategy for controlling leaf diseases [26,27]. Biostimulators may promote mycorrhizal fungi growth but also indirectly contribute to the development of fungal plant pathogens, as revealed in our results. There are numerous studies showing the different effects of fungicides application on *Fusarium* spp. reduction; however, in field experiments, the efficacy of fungicides is often examined exclusively without herbicidal protection. It was noticed that 250 mg L^{-1} of propiconazole [28], metconazole (1 L ha^{-1}), tebuconazole (1 kg ha^{-1}), prochloraz (1.1 L ha^{-1}), and prothioconazole (0.8 L ha^{-1}) [29] singly applied are effective fungicides against *Fusarium* spp. Our results indicate that the use of a sulfonylurea herbicide (26.5 g ha^{-1}) combined with propiconazole and cyproconazole (a total of 200 mL ha^{-1}) and spiroxamine, tebuconazole, and triadimenol (a total of 600 mL ha^{-1}) fungicides is the best strategy to reduce *Fusarium* spp. in wheat under field conditions (Figure 2). Apart from fungicides, sulfosulfuron singly applied also reduced the amount of *Fusarium* spp. due to the reduction of weed number and humidity, which favors fungi development. Interestingly, glyphosate-based herbicides can intensify the colonization of crops by fungi due to glyphosate interactions with the metabolic pathways of selected microorganisms [30]. However, Sanyal et al. [31] observed a lower amount of *Fusarium* spp. on green pea after glyphosate treatment. This indicates that the severity of fungal diseases is dependent on the type of herbicide, plant species, and phytoalexins, which can interact with microorganisms.

In 2020, the lowest total precipitations and *Fusarium* spp. concentrations, but also the greatest level of mycotoxins, were observed, which indicates that, in contrast to some studies [32,33], *Fusarium* secondary metabolites are secreted in the climatic conditions (especially humidity) that are unfavorable for fungal growth [34]. Moreover, it can be assumed that despite a lower *Fusarium* spp. severity in drier years, mycotoxins concentration is higher in cereal cultivation. This shows that abiotic environmental stress conditions probably intensified the expression of mycotoxin co-products in fungal biosynthesis pathways. To the best of our knowledge, this is the first study indicating lower mycotoxins contamination due to herbicidal protection combined with a humic biostimulator (Table 3, Figure 3). It was also previously indicated that herbicide MCPA can decrease the concentration of 3-AcDON and ZON mycotoxins in wheat grain [8]. In addition to our results, it was noticed that prothioconazole (87.5 g ha^{-1}), azoxystrobin (60 g ha^{-1}), and fluxapyroxad (40 g ha^{-1}) limit mycotoxins concentration in wheat grain [35]. Moreover, treatments based on epoxiconazole (0.5 L ha^{-1}), pyraclostrobin (0.5 L ha^{-1}) and, mancozeb (1 kg ha^{-1}) did not reduce mycotoxins concentration or even induce DON amount [36]. We concluded that treatment including sulfosulfuron (750 g L^{-1}), propiconazole (250 g L^{-1}), cyproconazole (80 g L^{-1}), spiroxamine (250 g L^{-1}), tebuconazole (167 g L^{-1}), and triadimenol (43 g L^{-1}) (H + F1 + F2) is the best strategy to most effectively reduce mycotoxins accumulation in cereals; however, humic biostimulator addition to the herbicidal protection can also decrease mycotoxins concentration. Our results indicate that the level of particular mycotoxins in different years may be diverse depending on the type of treatment, climatic condition during crop season, or occurrence of variable *Fusarium* spp. and other fungi which secrete different profiles of secondary metabolites [37].

Moreover, it was noticed that ergosterol concentration increases during grains ageing and can be dependent on precipitation [30]; however, our study did not confirm the relation

of ergosterol to climatic conditions, though a slight positive correlation with mycotoxins content was indicated (r = 0.32) during the four-year study. The number of weeds was the highest in 2017 (up to 84 pieces per 1 m^2) and was caused by the greatest precipitation (293 mm). The biostimulator also contributed to a greater weed infestation compared to treatments without its use. This results from the non-selective action of biostimulators, which promote wheat growth but also indirectly contribute to the increased development of weeds [38]. Similar to some other studies [39], biostimulator addition is not effective in weed control as it often causes higher weed infestation and, as a consequence, the development of fungal diseases. However, in contrast to amidosulfuron, iodosulfuron, mefenpyr-diethyl, and propoxycarbazone-sodium herbicides combined with prochloraz, tebuconazole, and proquinazid [40,41], sulfosulfuron protection combined with fungicides was the most effective in weed infestation reduction compared to single herbicide application. However, weed infestation was not observed after the application of biostimulator ComCat based on plant extracts [42].

In contrast to a "from field to fork" strategy, more complex chemical protection including herbicides and fungicides can contribute to better wheat parameters. As indicated by Hossard et al. [43], the reduction of pesticides dose by 50% caused a lower wheat yield (up to 10%); therefore, efficient programs should be developed which are based on different classes of biostimulators and could replace or modulate the positive effects of pesticides on target plants' protection. Moreover, integrated plant management with crop rotation can also increase yield and contribute to the reduction of weed infestation. Brankov et al. [44] confirmed that crop rotation with winter wheat caused a higher yield and lower weed infestation in maize cultivation. Moreover, the tillage system of crop production reduced disease severity and the concentration of the DON mycotoxin in wheat with a maize rotation system [45].

Many studies aim to minimize diseases' severity and achieve the greatest yields in different protection strategies and diversified climatic conditions. However, there are not many reports showing the economic proficiency of the results obtained in agricultural practice [46]. The findings indicated in this study enabled a compromise between the most desired protection strategy with high quality yield, low diseases severity, mycotoxins concentration, and the most beneficial economic profitability. Therefore, it can be assumed that complex protection including sulfonylurea herbicides combined with morpholine and triazole fungicides and a humic biostimulator best meets these conditions.

5. Conclusions

Optimal chemical/biostimulator protection is crucial for obtaining safe and healthy wheat grain with low fungal diseases severity and mycotoxins level. The results of our research are a response to the current problems in the cultivation of wheat affected by fungal diseases and weeds, and therefore may have a universal character in the countries of Central and Eastern Europe. Despite the same chemical/biostimulator protection, differences related to climatic conditions of East-Central Europe in yield, disease severity, *Fusarium* spp. concentration, and mycotoxins level were observed in individual years of the study. Complex chemical treatment, including a sulfonylurea herbicide, morpholine, and triazole fungicides, is the most effective strategy to obtain high yield and wheat grain of good quality with a low level of fungi and mycotoxins contamination. Furthermore, the addition of a humic biostimulator reduced mycotoxins level in herbicidal treatment, but negative effects, such as fungal diseases and higher weed infestation, were observed in the treatments enriched with a biostimulator. The effectiveness of the positive action of the humic biostimulator depends on climatic conditions. Exclusive sulfonylurea treatment had a relatively high disease severity and mycotoxins level. However, considering economic profitability, herbicidal treatment combined with fungicides and a biostimulator is the most valuable and has the greatest saved production value. These research findings indicated that humic biostimulators can support chemical treatment in the reduction of

mycotoxins and fungal diseases in particular climatic conditions and could be implemented in agricultural practice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12020258/s1. Figure S1: Chromatogram of mycotoxin standards (A,C) and wheat sample under herbicidal treatment in 2020 (B,D), Table S1: Results of ANOVA in examined parameters during four years of the study.

Author Contributions: Conceptualization, B.L.; methodology and formal analysis, P.I., R.K., P.K., N.K. and Y.D.; investigation, B.L., P.I. and P.K.; writing—original draft preparation, P.I.; writing—review and editing, B.L.; supervision, B.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are available in a publicly accessible repository.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

The analytical method of mycotoxins determination in wheat grain by liquid chromatography coupled with tandem mass spectrometry is detailed herein.

Appendix A.1. Wheat Sample Preparation

Before analysis, samples were ground and stored at −16 °C. Sample was weighed (5 g) in a centrifuge tube and extracted with 10 mL 1% CH_2O_2 in C_2H_3N (1:1, *v*/*v*). Next, 4 g $MgSO_4$, 1 g NaCl, 1 g $Na_3C_6H_5O_7$, and 0.5 g HOC(COOH)(CH2COONa)$_2$·1.5 H_2O were added. The tubes were shaken (1 min), vortexed (1 min), and centrifuged for 5 min at 4500 rpm. Supernatant was collected in another tube (15 mL) and stored at −60 °C for 30 min. The extract (5 mL) was transferred to a centrifuge tube with 150 mg $MgSO_4$, 25 mg PSA, and 25 mg C15. The samples were vortexed (1 min) and centrifuged at 4500 rpm for 10 min. The final extract (1 mL) was filtered through a 0.45 µm hydrophilic PTFE filter to the autosampler vial and subsequently analyzed using LC–MS/MS.

Appendix A.2. LC–MS/MS Analysis

A liquid chromatography system (Eksigent Ultra LC-100; Eksigent Technologies, Dublin, CA, USA) was used at a flow rate of 0.5 mL min^{-1} without splitting. A KINETEX XB C18 1.7 µm, 2.1 × 50 mm (Phenomenex) column was applied and heated to 40 °C during the analysis. A volume of 10 µL was injected into the LC–MS/MS device. The binary mobile phase was composed of H_2O + 0.5% CH_2O_2 + 5 mM NH_4HCO_2 (phase A) and CH_3OH + 0.5% CH_2O_2 + 2 mM NH_4HCO_2 (phase B). The following gradient elution was established: 95% A and 5% B (1 min), rising gradually to 10% A and 90% B (6 min) and held for 3 min. Next, the composition of the mobile phase changed to the initial condition and was held for 3 min. Mass spectrometric analysis was conducted using a MS/MS 6500 QTRAP system (AB SCIEX Instruments, Foster City, CA, USA), coupled with an electrospray ionization source (ESI). The capillary voltage was set at 5000 V for the positive and −4500 V for the negative ion mode. The turbo heater's temperature of 400 °C was maintained. Nitrogen was applied at a pressure of 60, 50, and 30 psi, respectively, as the nebulizer gas (GS1), auxiliary gas (GS2), and curtain gas (CUR). Additionally, nitrogen was also used as the nebulizer and collision gas. The multiple reaction monitoring mode

(MRM) was conducted to determine all mycotoxins. For each mycotoxin, the precursor ion and two product ions were determined: one product ion for quantification and one for qualification (Table A1).

Table A1. LC–MS/MS parameters for the determination of mycotoxin in wheat.

Mycotoxin	Quantification				Confirmation				EP [5] (V)
	MRM Transition [1] *m/z*	DP [2] (V)	CE [3] (V)	CXP [4] (V)	MRM Transition [1] *m/z*	DP [2] (V)	CE [3] (V)	CXP [4] (V)	
POSITIVE ION MODE									
3-AcDON	339 > 231	70	17	16	339 > 203	70	21	55	5
15-AcDON	356 > 137	46	21	8	339 > 137	126	15	10	5
DAS	367 > 307	58	9	55	367 > 349	58	11	24	5
FUM B1	722 > 334	165	53	20	722 > 352	165	51	22	5
FUM B2	706 > 336	135	49	20	706 > 318	135	53	18	5
FUM B3	706 > 336	51	35	17	706 > 354	51	29	21	5
HT-2	442.2 > 263	61	17	30	442.2 > 215.1	61	19	28	5
NEO	400 > 185	56	27	12	400 > 215	51	17	14	5
T-2	484.2 > 305.2	76	19	20	484.2 > 215.1	76	25	26	5
NEGATIVE ION MODE									
DON	355.1 > 295.1	−45	−14	−7	355.1 > 265.1	−45	−20	−15	−5
FusX	413.1 > 353	−50	−14	−7	413.1 > 263	−50	−20	−17	−5
NIV	371.1 > 311.1	−50	−14	−7	371.1 > 281	−50	−20	−17	−5
ZON	317.1 > 131.1	−85	−38	−9	317.1 > 175	−85	−32	−11	−5

[1] Multiple reaction monitoring mode; [2] declustering potential; [3] collision energy; [4] cell exit potential; [5] entrance potential.

Appendix A.3. Validation Protocol

During the validation study of the analytical method, the following parameters were determined: the accuracy (recovery), precision, limit of detection (LOD), limit of quantification (LOQ), linearity, deviation of the back-calculated concentration (DEV), matrix effect (ME), and uncertainty (U).

Four enrichment levels (LOQ, 10, 50, and 200 µg kg^{-1}) were prepared to the evaluate accuracy (average recovery) and precision (relative standard deviation, RSD). The range of recoveries of 70–120% was acceptable with a repeatability of ≤20% (RSD ≤ 20%). Recovery rates outside the range of 70–120% were accepted if consistent (RSD ≤ 20%), but the recovery should not be lower than 30% or above 140% [23]. As seen in Table A2, most of the mycotoxins presented satisfactory recoveries within the range between 70% and 120%, with RSD values ≤20%, i.e., compliant with the SANTE criteria (Table A2). Only toxin T-2 showed a recovery slightly above 120% (132%) at the lowest spiking level of 0.1 µg kg^{-1} with an acceptable RSD value (15%).

The limits of detection (LOD) and limits of quantification (LOQ) were calculated for a signal-to-noise ratio (S/N) of 3 and 10, respectively, by spiking the wheat sample at the concentration of 5 µg kg^{-1}. The determined values were included in Table A2.

Matrix-matched calibration at six different concentration levels of LOQ—5, 10, 25, 50, 200 and 500 µg kg^{-1}—was used to study linearity, and DEV was calculated as follows: $\%DEV = (C_{measured} - C_{true}) \times 100/C_{true}$) [23]. The method used in this study achieved a good linearity with a DEV lower than ± 20% (between −16% and 18%) (Table A3).

To estimate the matrix effects for all analyzed analytes, the same calibration curves in the pure solvent (acetonitrile) were compared with the calibration curves obtained during the matrix-matched calibration. MEs were calculated with the following formula: $\%ME = ((\text{peak area}_{(\text{matrix standard})}/\text{peak area}_{(\text{solvent standard})}) - 1) \times 100$. The positive values mean the matrix effect is enhanced, while the negative values mean the matrix effect is suppressed. If the matrix effects fell below −50% or above +50%, it was considered a strong effect, while it was considered a soft (−20% < MEs < 20%) or a medium effect when the values were between −50% < MEs < −20% and 50% > MEs > 20%. The sample extraction and purification procedure used in this study allowed a soft matrix effect to be obtained for all tested mycotoxins. The obtained values ranged from −16% to 19% (Table A2). The uncertainty of measurement was calculated individually for each analyte and estimated based on the data obtained in the validation study. The relative expanded uncertainty

was estimated by applying a "top-down" empirical model with coverage factor k = 2 at a 95% confidence level. In all cases, the expanded uncertainty was acceptable and ranged from 11% to 21% (Table A2), and was less than the default value of 50% recommended by SANTE.

Table A2. Average recoveries, RSDs, LODs, LOQs, and expanded uncertainties U, % (k = 2, confidence level 95%) of mycotoxin in wheat.

Mycotoxin	LOD [1] (μg kg^{-1})	LOQ [2] (μg kg^{-1})	Recovery (RSD [3]) (%)				ME [4] (%)	U [5] (%)
			1st Level = LOQ	2nd Level 10 (μg kg^{-1})	3rd Level 50.0 (μg kg^{-1})	4th Level 200.0 (μg kg^{-1})		
15-AcDON	0.3	1	95 (14)	93 (10)	77 (4)	94 (4)	−13	11
3-AcDON	0.4	1	94 (15)	112 (14)	86 (7)	72 (14)	−10	13
DAS	0.2	0.5	86 (7)	82 (8)	86 (7)	76 (6)	14	19
DON	1.5	5	73 (18)	72 (7)	78 (8)	98 (7)	−5	11
FUM B1	0.3	1	112 (3)	101 (4)	101(4)	78 (8)	8	20
FUM B2	0.2	1	96 (6)	95 (5)	94 (6)	98 (5)	11	21
FUM B3	0.25	1	65 (7)	66(5)	69 (5)	100 (4)	−6	14
FusX	0.6	2	77 (3)	79 (6)	78 (7)	74 (6)	−9	11
HT-2	0.3	1	75 (9)	76(8)	79 (6)	82 (6)	−16	17
NEO	0.65	2	70 (7)	77 (14)	80 (7)	81 (5)	19	13
NIV	0.35	1	94 (13)	82 (11)	106 (4)	88 (5)	11	14
T-2	0.03	0.1	132 (15)	99 (15)	103 (5)	84 (6)	−7	13
ZON	0.35	1	90 (5)	93 (7)	100 (4)	94 (5)	12	14

[1] Limit of detection; [2] limit of quantification; [3] relative standard deviation; [4] matrix effect; [5] uncertainty.

Table A3. Regression equations and deviation of the back-calculated concentration (DEV) of mycotoxins in wheat.

Mycotoxin	Regression Equation	DEV (%) LOQ [1]	DEV (%) 5.0 (μg kg^{-1})	DEV (%) 10.0 (μg kg^{-1})	DEV (%) 25.0 (μg kg^{-1})	DEV (%) 50.0 (μg kg^{-1})	DEV (%) 200.0 (μg kg^{-1})	DEV (%) 500.0 (μg kg^{-1})
15-AcDON	y = 4.1797x − 0.0101	17	13	−7	3	4	−1	1
3-AcDON	y = 1.8211x − 0.0060	5	9	2	1	10	2	3
DAS	y = 1.2567x − 0.0014	−11	6	−6	14	−6	5	−2
DON	y = 8.0287x + 0.0005	-	8	8	−15	12	1	−1
FUM B1	y = 1.4562x + 3.7151	−3	1	5	−3	3	2	−1
FUM B2	y = 1.8879x + 2.6171	−4	11	10	3	−4	−2	−1
FUM B3	y = 0.0019x − 0.0071	−3	12	−6	4	−7	10	−1
FusX	y = 0.0021x − 0.0281	11	8	18	−8	9	3	−4
HT-2	y = 4.4066x − 1.9306	17	3	1	2	3	3	2
NEO	y = 1.8879x + 2.6171	−4	2	5	−1	−2	6	−1
NIV	y = 0.0019x − 0.0071	−3	9	11	−16	−7	11	−1
T-2	y = 0.0030x − 0.0109	−10	−2	6	−5	3	4	−4
ZON	y = 5.9983x − 1.0007	9	6	−7	5	−9	−4	−1

[1] Limit of quantification.

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