

Special Issue Reprint

Strategies to Improve the Security and Nutritional Quality of Crop Species

Edited by
Grażyna Podolska and Anna Szafrńska

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Strategies to Improve the Security and Nutritional Quality of Crop Species

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Article

The Influence of *Fusarium culmorum* on the Technological Value of Winter Wheat Cultivars

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Abstract: The research hypothesis assumes that *Fusarium culmorum* infection affects the baking value of wheat. The aim of the research was to determine the effect of the cultivar on the rheological properties of wheat dough in response to *Fusarium culmorum* infection of wheat. A two-factor experiment conducted during the 2018–2020 growing seasons in Osiny, Poland, was set up using the completely randomized block design with three replications. The first factor was winter wheat cultivars (six cultivars), while the second factor was inoculation (two levels—*Fusarium culmorum* and distilled water—control). The immunoenzymatic ELISA method was used to determine the content of deoxynivalenol (DON) in grain. The DON content in the grain varied between cultivars. *Fusarium culmorum* inoculation resulted in an increase in protein, ash content, and flour water absorption, changes in dough rheological properties, and a decrease in the sedimentation index. Inoculation also caused negative changes in starch properties. The observed interaction between *Fusarium culmorum* inoculation and cultivars in shaping the qualitative parameters and rheological properties of the dough indicates that there are wheat cultivars less susceptible to *Fusarium* infection, which do not show any significant changes as a result of infection.

Keywords: artificial inoculation; food safety; *Fusarium culmorum*; grain quality; deoxynivalenol; rheological properties; wheat

1. Introduction

Wheat is one of the most important crops in the world, ranking as the third largest in terms of production, after sugar cane and maize. In 2022, global wheat production reached 808 million tons, cultivated on an area of 219.2 million hectares [1]. The data indicate that over 50% of wheat harvests are used by food enterprises for production of flour, bread, breakfast cereals, pasta, and others [2]. Wheat intended for bread production must meet specific quality indicators that determine its suitability for flour production and baking. For this purpose, the grain undergoes various analyses, which provide information on the quantity and quality of gluten, the quality of the protein complex (sedimentation index), the activity of amylolytic enzymes (falling number), and the rheological properties of the dough [3].

The raw material delivered to flour mills must not only meet the requirements for baking quality but also be completely safe for human health. The quality of the raw material, collected from the field, is influenced by the presence of physical, chemical, or biological

contaminants. Chemical contaminations, such as pesticide residues, heavy metals, and nitrites, are not acceptable in final food products and feed. Grain infection by *Fusarium* spp. causing *Fusarium* head blight (FHB) may be indicated by high levels of mycotoxins.

Mycotoxins are a group of harmful compounds, which are secondary metabolites of filamentous fungi. These substances can have strong toxic, mutagenic, or teratogenic effects. Secondary metabolites are organic compounds produced by organisms, such as plants, fungi, and bacteria, that are not directly involved in their normal growth, development, or reproduction. The presence of mycotoxins in food poses a potential threat to human health. It is estimated that about 25% of global cereal production is contaminated with these metabolites, leading to a deterioration in the health quality of grain and its elimination from consumption and feed [4]. To ensure consumer safety, the European Commission has established unified standards and legal regulations regarding the maximum levels of some of the most important mycotoxins in cereal grains and products. The maximum level of DON is set at 1000 $\mu\text{g kg}^{-1}$ for unprocessed cereals, except for durum wheat with a limit of 1500 $\mu\text{g kg}^{-1}$ and oats with a limit up to 1750 $\mu\text{g kg}^{-1}$ [5]. For zearalenone (ZEN), the maximum level for these products is 100 $\mu\text{g kg}^{-1}$. The maximum levels of mycotoxins in cereal-based products are lower. The DON level in flour, pasta, and bran must not exceed 600 $\mu\text{g}\cdot\text{kg}^{-1}$, in bread 400 $\mu\text{g}\cdot\text{kg}^{-1}$, and in products for children 150 $\mu\text{g}\cdot\text{kg}^{-1}$. Similarly, the ZEN level in flour and bran must not exceed 75 $\mu\text{g}\cdot\text{kg}^{-1}$, and in bread it must not exceed 50 $\mu\text{g}\cdot\text{kg}^{-1}$ [6].

Wheat cultivation is primarily aimed at producing grain for consumption, especially for flour and bread production. Wheat grain is rich in various components that differ in molecular structure, physicochemical, and functionality. Its dry mass consists of 60–70% starch, 10–17% proteins, 3–6% sugars, 2–2.5% lipids, vitamin E, B group vitamins (riboflavin, niacin, thiamine), dietary fiber, 1.4–2.3% minerals (Cu, Mg, Zn, P, and Fe), and other phytochemical compounds [7,8]. The chemical composition of wheat grain is influenced by environmental conditions, agricultural practices, and genotype [7].

Fusarium fungal infection of grain can lead to biochemical changes and structural alteration in the grain [9]. The extent of these changes depends on the level of fungal invasion [10]. Infected grain may have fungal spores on its surface, and its internal structure may contain mycotoxins. Infested grain shows a reduction in the thickness of the aleurone layer, altering the proportion of seed coat to endosperm, which results in a decrease in thousand kernel weight and an increase in ash content in whole-grain flour [11–13]. A positive correlation between the degree of *Fusarium culmorum* infection and the ash content, which includes potassium and calcium salts in wheat grain, was previously reported [14,15].

Increased activity of amylase, protease, and other enzymes responsible for the degradation of non-starch polysaccharides in infected samples was also observed [16–19]. Fungal proteases produced by *Fusarium culmorum* are active in a wide range of temperatures (10–100 °C) and pH values (4.5–8.5), potentially impairing dough functionality and bread quality throughout processing. Kreuzberger et al. [17], in an in vitro study, observed a clear effect of *Fusarium graminearum* proteases on glutenin and gliadin fractions, with a higher preference for glutenins, which are responsible for dough elasticity and viscosity. *Fusarium*-infected ears, during kernel growth, may show little visible damage or macromolecules inside the kernel but still contain high levels of DON [20]. The fungal infection may destroy the cellular structures of the starchy endosperm, damaging starch granules. These compositional may reduce flour quality, resulting in a low falling number [21]. The presence of *Fusarium* leads to starch degradation, which correlates with the activity of α -amylase in the grain and a decrease in the falling number value [9,22,23].

Starch damage caused by *Fusarium* may contribute to increased water absorption in the samples [18]. The quality of wheat flour primarily depends on gluten proteins, which play an important role in carbon dioxide retention, dough development, and baking quality [24]. *Fusarium* fungi and their toxins can affect both protein quantity and disturb the mutual proportions of individual protein fractions, thereby impacting the quality of the raw material and the final product [25]. In some cultivars (Greina, Lona, and Brusino), the sedimentation index decreases after *Fusarium* infection, indicating an effect on the protein quantity and quality, while no such negative changes were observed in others. Studies indicate that *Fusarium* decreases gluten quantity and quality, as evidenced by the reduction in the Zeleny sedimentation index in *Fusarium*-infected grain compared to healthy seeds [14,21]. The reduction in the quality of gluten proteins due to *Fusarium* inoculation also affected the rheological properties of wheat flour dough. The increase in protease activity found in wheat flour infected by *Fusarium* had a significant effect on the increase in dough softening and decrease in dough stability of flour [13,14,18]. However, the *Fusarium* infection did not influence the water absorption of flour [14,18]. The deterioration of qualitative characteristics and rheological properties of the dough made by infected flour was also noted previously. *Fusarium* infection also negatively impacted dough energy, maximum tensile strength, and the strength to extensibility ratio, reducing these parameters significantly. Additionally, some authors observed a 20-fold increase in the gliadin to glutenin ratio in flour samples with a DON content of $3.98 \text{ mg} \cdot \text{kg}^{-1}$, resulting in reduced bread volume [13,26]. Contrary to the abovementioned results, other authors noted an increase of loaf volume under *Fusarium* infection regarding the dose of *Fusarium* infection and different bread production technologies [18,27]. Limited studies using Mixolab, which allows for the assessment of the protein–starch complex in a single test, showed that *Fusarium* spp. infection of the grain led to a reduction in the rheological properties of dough. Other research showed that an increasing intensity of *Fusarium* spp. contamination had negative effects on protein and mainly on the starch part of grain which was noted on the Mixolab curves [13]. The authors of [13,28] suggested that the cause may be the degree of starch damage granules in the inoculated samples, which affects the dough characteristics, making it stickier and potentially resulting in poor baking quality.

The research hypothesis posits that *Fusarium* infection and mycotoxins in grain and flour affect wheat's baking quality, causing changes in both starch and protein structure. These effects are cultivar-dependent. Therefore, the aim of this research is to determine the impact of cultivar on the rheological quality of wheat dough in response to stress caused by *Fusarium* infection of wheat.

2. Materials and Methods

A two-factor experiment was established using the completely randomized block method in three replications. The factors were winter wheat cultivars and spray. In the experiment, six winter wheat cultivars were used, with each cultivar being treated with either *Fusarium culmorum* inoculation or distilled water (as a control sample).

Plant material. The research work concerned winter wheat cultivars representing two technological groups approved by the Polish National List of Agricultural Plant Varieties issued yearly by COBORU (the Research Centre for Variety Testing). The individual cultivars differed in morphological structure, utility features, genotype and place of cultivation (Table 1). Group A comprises quality bread cultivars, namely: 'Legenda', 'Pokusa', 'Tonacja', and 'Sailor'. Group B comprises the following bread cultivars: 'Muszelka' and 'KWS Ozon'.

The cultivars differed in earliness and the earing stage; the fastest were cv. 'Muszelka', 'Pokusa', 'Sailor', and the latest was cv. 'Tonacja'. The maturity stage was reached the fastest by cv. 'Muszelka' and 'Pokusa' (202 days from 1 January), and the latest by: cv.

‘KWS Ozon’ and ‘Sailor’ (203 days from 1 January). The cultivars differed in resistance to *Fusarium* head blight (FHB). Resistance is estimated by the Central Research Centre for Cultivar Testing on a nine-point scale, where 1 means susceptible and 9 completely resistant. The cv. ‘Legenda’ was characterized by the highest resistance (7.9) and the cv. ‘Muszelka’ (6.5) showed the highest susceptibility. Moreover, the cultivars differed in height. The height difference was 35 cm. The tallest cultivar was cv. ‘Legenda’ (115 cm) and the shortest was cv. ‘Muszelka’ (80 cm) (Table 1).

Table 1. Characteristics of winter wheat cultivars.

Cultivar	Breeding Place	Resistance to FHB *	Height (cm)	Wheat Quality Group	Flowering, Maturing (Days from 1st January)
KWS Ozon	KWS Lochow GmbH	7.1	83	B	151 203
Legenda	Poznańska Plant Breeding	7.9	115	A	151 203
Muszelka	DANKO Plant Breeding	6.5	80	B	150 202
Pokusa	Strzelce Plant Breeding	7.9	97	A	150 202
Tonacja	Strzelce Plant Breeding	7.8	104	A	152 204
Sailor	DANKO Plant Breeding	7.7	101	A	150 203

* FHB—Fusarium head blight.

Field trials. The field experiment was conducted in 2018/2019 and 2019/2020 growing seasons at the Experimental Station in Osiny (51°35′, 21°55′), belonging to the Institute of Soil Science and Plant Cultivation—State Research Institute (IUNG-PIB) Puławy, Poland. The experiment was established on pseudopodzolic soil, class IIIb, agricultural soil suitability complex—very good rye complex, with a pH of 6.1. The mineral content in the soil was: extractable phosphorus (P: 12.6 mg kg^{−1}), exchangeable potassium (K: 15.3 mg kg^{−1}), magnesium (Mg 4.9 mg kg^{−1}). The forecrop was winter rape. The plot size was 24 m², 11.25 m² was designed for harvest. In autumn, after harvesting the forecrop, pre-sowing of crops was carried out—cultivation with a disc cultivator, ploughing and cultivation with a seed drill. Wheat sowing was performed on optimal dates in subsequent years of the experiment: 2 October 2018 and 7 October 2019, respectively. Sowing density was 450 grains per m². The plots were fertilized with: N (160 kg ha^{−1}), P (70 kg ha^{−1}), and K (105 kg ha^{−1}). Nitrogen fertilizer was applied three times in individual vegetation stages: beginning of vegetation (BBCH 23)—70 kg N ha^{−1}, stem shooting (BBCH 32)—60 kg N ha^{−1}, full earing (BBCH 53)—30 kg N ha^{−1}. Plants were protected against weeds, diseases, and pests using Bizon 400 L (active substance: tralkoxydim) 1.0 l·ha^{−1}; Mustang Forte 195 SE (active substance: florasulam, aminopyralid, 2.4D) 0.5 l ha^{−1}; Unix 75WG (active substance: cyprodinil) 0.6 kg ha^{−1}; Fury 100 EC (active substance: zeta-cypermethrin) 0.1 L ha^{−1}. Harvesting was carried out at full maturity on 20 July 2019 and 1 August 2020.

Weather conditions. The weather conditions during the wheat growing period, including the heading (May), flowering (June), and maturity (July) stages, varied across different growing seasons (Table 2). In 2019, air temperature was higher in May and June by 1.8 °C and 3.3 °C, respectively compared to the 2020 harvest year. Precipitation in the mentioned months of 2019 was lower by 26.6 mm, 150.8 mm, and 15.9 mm, respectively, compared to 2020. The high air temperature in June and July, together with very low precipitation, led to the occurrence of drought. These were unfavorable conditions for the growth and development of *Fusarium culmorum*.

Table 2. Weather conditions in the growing seasons.

Month	Temperature (°C)		Precipitation (mm)	
	Growing Season			
	2019	2020	2019	2020
March	5.5	4.5	22.7	25.3
April	9.6	8.5	35.5	11.9
May	12.9	11.1	86.1	112.7
June	21.7	18.4	38.7	189.5
July	18.6	18.6	33.9	49.8

Preparation of Fusarium culmorum inoculum and inoculation in the field. The inoculation suspension was obtained from grain infected with *Fusarium culmorum* spores, prepared at the Plant Breeding and Acclimatization Institute (IHAR)—National Research Institute in Radzików, Poland. To produce the inoculum, a *Fusarium culmorum* isolate forming deoxynivalenol was prepared according to the methodology followed in [29]. The isolate was incubated on autoclaved wheat grain in glass flasks for approximately 4 weeks and then irradiated with continuous UV light for 4 to 7 days at a temperature of about 18 °C. Then the grain infected with *Fusarium culmorum* was dried and stored at 4 °C until the inoculates were prepared for spraying. On the day of inoculation, grain with mycelium and conidial spores of *Fusarium culmorum* was soaked in sterile tap water for two hours and then filtered through gauze to obtain a spore suspension without mycelium fragments. After thoroughly mixing the spore suspension, the number of colony-forming units (CFU) was determined using a Thom counting chamber. The field experiment used artificial inoculation by spraying with *Fusarium culmorum*. It was performed during the flowering phase of the wheat cultivars (BBCH 65). The plants were sprayed manually with a suspension of spores prepared in the laboratory at a concentration of 5×10^5 CFU·mL⁻¹ at 100 mL per 1 m². The inoculation method used imitated the natural conditions of ear infection by *Fusarium* and allowed for determining type I resistance (resistance to infections) and type II resistance (resistance to the spread of the pathogen in tissues). Inoculations were performed in the late afternoon hours, when the temperature decreased and the relative air humidity increased. The experiment included a control, on which spraying with distilled water was used during the flowering period in order to maintain similar humidity conditions to the inoculated field.

Determination of deoxynivalenol in wheat grain. The immunoenzymatic ELISA method was used to determine the content of DON in wheat grains, using the AgraQuant test from RomerLabs® (Getzersdorf, Austria). The optical density of the samples was measured using a Stat Fax 303 Plus microplate reader (photometer) (Awareness Technology, Inc., Palm City, FL, USA) at a wavelength of 650 nm. According to the recommendation, the results were read within 20 min of adding the Red Stop solution. The standard curve was prepared by using a standard (optical density of mycotoxin concentration). For each analysis the correlation coefficient R (dependence of absorbance on A/c concentration) was obtained. According to recommendations, the results are considered reliable when the value of the R coefficient is not less than 0.996. Representative samples of grain were ground in a Laboratory Mill 3100 by Perten and stored at 2–8 °C until extraction. After adding the extractant (distilled water), the samples were shaken vigorously for 3 min. The extracts were filtered by passing at least 5 mL through a Whatman 1 filter. The estimation was carried out in accordance with AgraQuant standards in duplicate. The contents of DON in inoculated and uninoculated samples (2019) and the control samples (2020) were below the detection limit (LOD < 200 µg kg⁻¹), therefore, these values are not presented in the manuscript.

Technological quality analysis. The quality analysis was determined after harvest. The physical and chemical properties were tested in the samples obtained only from the 2020 harvest year. In 2019 *Fusarium* infection was not observed at a level that would affect the technological properties of the grain, therefore the data from this year was abandoned. The thousand kernel weight was determined according to ISO 520:2010 [30]. A grinder (FN 3100, Perten Instruments AB, Hägersten, Sweden) was used for preparation of whole-grain flours, and next the following quality parameters were marked: moisture content (determined according to ISO 712:2009) [31], protein content (ISO 20483:2013) [32], gluten quantity (ISO 21415-2:2015) [33], Zeleny sedimentation index (ISO 5529:2007) [34], and ash content (ISO 2171:2023) [35] were determined in order to evaluate the technological quality of tested wheat cultivars. Wheat flour samples for the Zeleny sedimentation test were prepared by using a Sedimat laboratory mill (Brabender GmbH & Co. KG, Duisburg, Germany).

Rheological properties of a dough. Mixolab ChopinWheat+ Protocol according to ISO 17718:2013 [36] (KPM Analytics, Villeneuve-la-Garenne, France) was used to analyze the rheological properties of a dough. Grain was ground by an FN 3100 grinder (Perten Instruments AB, Hägersten, Sweden) to obtain whole-grain flour to perform the Mixolab test. The whole-grain flour samples (50 g) based on 14% moisture content were used to determine the water absorption of flour at the consistency of 1.1 ± 0.05 Nm. The mixing speed was 80 rpm, and bowl temperature was set at 30 °C. Protein properties related to water absorption, dough development time, stability, and weakening (C2, C1–C2, slope α) were determined. The starch properties correspond to starch gelatinization during the increase in the temperature from 30 to 90 °C (C3, C3–C2, slope β) and enzymatic activities (C4, C3–C4, slope γ) and retrogradation (C5, C5–C4) were also evaluated. The following Mixolab Profiler indexes were evaluated to better characterize the baking quality potential of tested cultivars: water absorption, mixing, gluten+, viscosity, amylase, and retrogradation.

Statistical analyses. The results were statistically analyzed by the one-way analysis of variance (ANOVA) with a subsequent Tukey's HSD test with the significance level of $p < 0.05$ to compare the means of control treatments and inoculations with *Fusarium culmorum*. The second factor was the wheat cultivar used. Principal component analysis (PCA) was performed to reduce the dimensionality of the data and represent the samples in a new coordinate system. All results were analyzed using Statistica software (v. 13.3, StatSoft, Tulsa, OK, USA).

3. Results

Wheat grain samples from the 2020 harvest year showed significant infection by *Fusarium* spp. and were also characterized by relatively high DON content. Variation in DON levels and basic quality parameters were observed. These characteristics are essential for assessing their suitability for grain processing for consumption. The results of the analysis of variance (Table 3) indicated that both the cultivars and *Fusarium* inoculation significantly affected the measured technological parameters of wheat grain. However, *Fusarium* inoculation had no significant effect on the gluten content. Table 3 does not include the results of wheat cultivars harvested in 2019 because no ear infection was observed, which was confirmed by DON determinations below the LOD. Additionally, no difference was found in the amount of gluten and the Zeleny sedimentation index between inoculated and control samples.

Table 3. Significance of effect in ANOVA for wheat samples from the 2020 harvest.

Parameter/Factor	A (Cultivar)	B (Inoculation)	A × B	V (%)
Thousand kernel weight	***	***	*	6.28
Protein content	***	**	***	3.29
Gluten content	***	n.s.	***	4.43
Zeleny sedimentation index	***	***	***	4.24
Ash content	***	***	***	7.55
Water absorption	***	***	***	3.49
Time T1	***	***	***	4.45
Stability	***	***	***	5.36
Slope α	***	***	***	3.85
Protein weakening, C2	**	***	**	5.63
C1–C2	***	***	***	4.40
Slope β	***	***	***	4.02
Slope γ	***	***	***	7.92
C3	***	***	***	3.44
C4	***	***	***	2.54
C5	***	***	***	4.83
C3–C2	***	***	***	3.47
C3–C4	***	***	***	2.54
C5–C4	***	***	***	3.12

Abbreviation: *—significant at $p < 0.05$; **—significant at $p < 0.01$; ***—significant at $p < 0.001$; n.s.—non-significant; V—coefficient of variation.

3.1. Deoxynivalenol Content in Wheat Grain

The deoxynivalenol (DON) content of individual grain wheat cultivars varied across different years. Grain samples harvested in 2019 were characterized by a DON level below the limit of detection (LOD) of $200 \mu\text{g}\cdot\text{kg}^{-1}$. In 2020, the grain of the tested wheat cultivars contained higher levels of DON compared to 2019. The lowest amounts of DON were accumulated in the cv. ‘Sailor’ ($6847 \mu\text{g}\cdot\text{kg}^{-1}$), cv. ‘Pokusa’ ($7477 \mu\text{g}\cdot\text{kg}^{-1}$), and cv. ‘Legenda’ ($8790 \mu\text{g}\cdot\text{kg}^{-1}$), while the highest in cv. ‘Muszelka’ ($11,021 \mu\text{g}\cdot\text{kg}^{-1}$), cv. ‘KWS Ozon’ ($11,033 \mu\text{g}\cdot\text{kg}^{-1}$), and cv. ‘Tonacja’ ($11,513 \mu\text{g}\cdot\text{kg}^{-1}$) (Figure 1). In 2020, in uninoculated samples (control), DON content was below limit of detection ($200 \mu\text{g}\cdot\text{kg}^{-1}$).

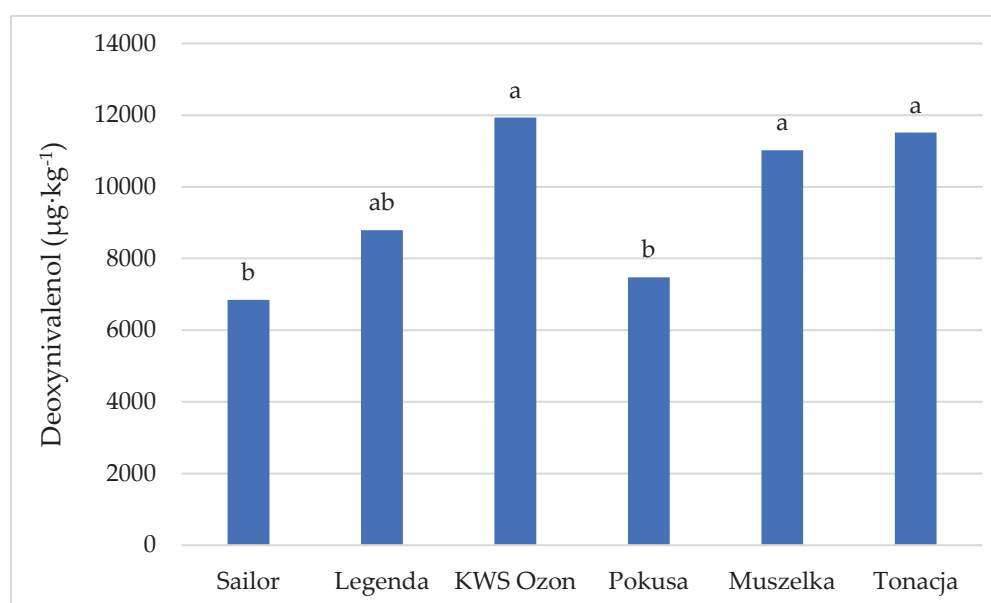


Figure 1. Deoxynivalenol (DON) content in wheat grain inoculated with *Fusarium culmorum* in 2020. The same letters indicate no statistically significant differences between the data, $p < 0.05$.

3.2. Quality Characteristics of Wheat Cultivars

The effect of *Fusarium culmorum* inoculation on technological features of grain is presented in Table 4. Thousand kernel weight (TKW) is related to grain yield, milling efficiency, and seedling vigor. TKW differed significantly between control samples of the cultivars, ranging from 38.93 g (cv. ‘Legenda’) to 47.51 g (cv. ‘KWS Ozon’). *Fusarium* inoculation caused a significant reduction in TKW in all cultivars, except for cv. ‘KWS Ozon’ (Table 4).

Table 4. The quality characteristics of winter wheat cultivars control and *F. culmorum*-infected samples.

Parameter	Inoculation	Cultivar						Mean
		Sailor	Legenda	KWS Ozon	Pokusa	Muszelka	Tonacja	
Thousand kernel weight (g)	Control	42.15 ^a	38.93 ^a	47.51 ^a	46.92 ^a	46.84 ^a	41.67 ^a	44.00 ^B
	<i>Fusarium</i>	38.52 ^b	33.24 ^b	43.52 ^a	43.73 ^b	42.15 ^b	36.17 ^b	39.56 ^A
	Average	40.3 ^{AB}	36.08 ^B	45.51 ^A	45.32 ^A	44.49 ^A	38.92 ^B	
Protein content (%)	Control	13.9 ^b	12.9 ^b	13.1 ^a	13.4 ^b	13.3 ^b	11.9 ^a	13.1 ^B
	<i>Fusarium</i>	14.8 ^a	16.4 ^a	13.2 ^a	14.3 ^a	14.1 ^a	12.4 ^a	14.2 ^A
	Average	14.4 ^A	14.6 ^A	13.1 ^D	13.9 ^B	13.6 ^C	12.1 ^E	
Gluten content (%)	Control	29.4 ^b	35.6 ^a	28.6 ^a	29.6 ^b	28.5 ^a	28.8 ^a	30.08 ^A
	<i>Fusarium</i>	30.5 ^a	34.2 ^b	27.4 ^a	31.8 ^a	28.7 ^a	30.0 ^a	30.43 ^A
	Average	30.0 ^A	34.9 ^A	28.0 ^B	30.7 ^A	28.6 ^B	29.4 ^A	
Zeleny sedimentation index (cm ³)	Control	48 ^a	45 ^a	52 ^a	55 ^a	47 ^a	46	48.8 ^A
	<i>Fusarium</i>	45 ^a	51 ^a	34 ^b	46 ^b	42 ^a	44 ^a	43.6 ^B
	Average	47.0 ^A	48.0 ^A	43.0 ^A	50.7 ^A	44.5 ^A	45.5 ^A	
Ash content (%)	Control	1.42 ^b	1.48 ^a	1.51 ^b	1.27 ^b	1.41 ^b	1.45 ^b	1.42 ^B
	<i>Fusarium</i>	1.56 ^a	1.59 ^a	1.91 ^a	1.71 ^a	1.71 ^a	1.58 ^a	1.68 ^A
	Average	1.49 ^B	1.53 ^B	1.71 ^A	1.49 ^B	1.56 ^B	1.52 ^B	

a,b,A–E—homogeneous groups, statistically significant differences at $p = 0.05$.

The protein content significantly depended on the cultivar. An interaction between *Fusarium culmorum* inoculation and cultivar was observed for protein content. Wheat samples inoculated with *Fusarium* had protein content in the range of 12.4% (cv. ‘Tonacja’) to 16.4% (cv. ‘Legenda’). The protein content in non-inoculated samples ranged from 11.9 (cv. ‘Tonacja’) to 13.9 (cv. ‘Sailor’). *Fusarium* inoculation caused a significant increase in protein content in grain, except for cv. ‘KWS Ozon’, where no effect was observed. The greatest increase in protein content was found in cv. ‘Legenda’, where the difference between inoculated and control samples was 3.5 percentage points. The difference for cv. ‘Sailor’, ‘Pokusa’, and ‘Muszelka’ ranged from 0.8 to 0.9 percentage points.

The gluten content in control wheat samples ranged from 28.6% (cv. ‘Muszelka’) to 35.6% (cv. ‘Legenda’) (Table 3). *Fusarium* inoculation caused a significant increase in gluten content in cv. ‘Sailor’ and cv. ‘Pokusa’ by 1.1, and 2.2 percentage points, respectively. In cv. ‘Muszelka’, ‘Tonacja’, and ‘KWS Ozon’, no significant differences were observed compared to the control samples.

The Zeleny sedimentation index values for flour from wheat grain samples ranged from 45 cm³ (cv. ‘Legenda’) to 55 cm³ (cv. ‘Pokusa’) (Table 3). *Fusarium* inoculation reduced the sedimentation index value on average by 5.2 cm³ across the cultivars. The highest reduction was observed in cv. ‘KWS Ozon’ (18 cm³) and cv. ‘Pokusa’ (9 cm³), while in cv. ‘Legenda’, there was an increase of 6 cm³.

Ash content in grain was influenced by both the cultivar and *Fusarium* inoculation (Table 3). Wheat samples were characterized by ash content in the range of 1.27% (cv. ‘Pokusa’) to 1.51% (cv. ‘KWS Ozon’). Infected grain showed significantly higher ash content compared to the control samples, with differences ranging from 0.11 percentage points (cv. ‘Legenda’) to 0.44 percentage points (cv. ‘Pokusa’).

3.3. Rheological Properties of the Protein Complex of Tested Wheat Cultivars

The rheological properties of whole-grain flour dough were studied using the Chopin-Wheat+ protocol of Mixolab (Chopin Technologies), which allows for the determination of both protein and starch properties of wheat flour in a single test.

Determination of flour water absorption is an important criterion for wheat flour, as it provides information about baking absorption, which is related to bread yield and quality of bakery products. In the presented studies, the water absorption of the flour samples from the cultivars ranged from 60.5% (cv. ‘Sailor’) to 62.9% (cv. ‘Tonacja’). A significant increase in this indicator was observed in all inoculated cultivars compared to the control samples. Flour obtained from grain infected with *Fusarium* showed higher water absorption, on average by 3.6 percentage points, compared to control grain samples (Table 5). The highest increases in this parameter were observed for cv. ‘Pokusa’ and cv. ‘Muszelka’ (4.6 and 5 percentage points, respectively).

Table 5. The rheological properties of dough obtained from winter wheat cultivar control and *F. culmorum*-infected samples.

Parameter	Inoculation	Cultivar						Mean
		Sailor	Legenda	KWS Ozon	Pokusa	Muszelka	Tonacja	
Water absorption (%)	Control	60.5 ^b	61.4 ^b	62.6 ^b	62.1 ^a	61.8 ^b	62.9 ^b	61.9 ^B
	<i>Fusarium</i>	64.6 ^a	63.4 ^a	66.4 ^a	66.7 ^b	66.8 ^a	64.9 ^a	65.5 ^A
	Average	62.6 ^A	62.4 ^A	64.5 ^A	64.4 ^A	64.3 ^A	63.9 ^A	
Time T1 (min)	Control	3.37 ^b	4.4 ^a	5.0 ^a	4.1 ^a	5.4 ^a	8.2 ^a	5.0 ^A
	<i>Fusarium</i>	4.0 ^a	2.6 ^b	1.9 ^b	3.9 ^a	1.2 ^b	3.2 ^b	2.8 ^B
	Average	3.66 ^B	3.50 ^B	3.47 ^C	4.00 ^B	3.25 ^D	5.65 ^A	
Stability (min)	Control	11.0 ^a	9.0 ^a	10.3 ^a	8.4 ^a	9.8 ^a	11.8 ^a	10.1 ^A
	<i>Fusarium</i>	7.7 ^b	8.2 ^b	7.0 ^b	6.2 ^b	5.3 ^b	5.2 ^b	6.6 ^B
	Average	9.3 ^A	8.6 ^A	8.6 ^A	7.3 ^B	7.6 ^B	8.5 ^A	
Slope α (Nm min ^{−1})	Control	−0.102 ^b	−0.088 ^a	−0.101 ^b	−0.083 ^b	−0.090 ^a	−0.091 ^b	−0.092 ^B
	<i>Fusarium</i>	−0.082 ^a	−0.091 ^a	−0.091 ^a	−0.070 ^a	−0.090 ^a	−0.060 ^a	−0.080 ^A
	Average	−0.092 ^B	−0.090 ^B	−0.096 ^B	−0.076 ^A	−0.090 ^B	−0.075 ^A	
Protein weakening, C2 (Nm)	Control	0.53 ^a	0.42 ^a	0.46 ^a	0.40 ^a	0.47 ^a	0.55 ^a	0.47 ^A
	<i>Fusarium</i>	0.21 ^b	0.22 ^b	0.17 ^b	0.18 ^b	0.15 ^b	0.13 ^b	0.18 ^B
	Average	0.36 ^A	0.32 ^B	0.31 ^B	0.29 ^C	0.31 ^B	0.33 ^B	
C1–C2 (Nm)	Control	0.58 ^b	0.66 ^b	0.63 ^b	0.67 ^b	0.62 ^b	0.55 ^b	0.62 ^B
	<i>Fusarium</i>	0.91 ^a	0.89 ^a	0.94 ^a	0.95 ^a	0.97 ^a	0.97 ^a	0.94 ^A
	Average	0.74 ^B	0.77 ^B	0.78 ^A	0.81 ^A	0.79 ^A	0.76 ^{AB}	

a,b,A–D—homogeneous groups, statistically significant differences at $p = 0.05$.

The protein characteristics of whole-meal flour samples were determined based on time T1 (dough development time), stability, and protein weakening (C2, C1–C2, slope α). *Fusarium* inoculation significantly influenced the protein characteristics of the tested winter wheat cultivars.

Dough development time (T1), which is primarily related to protein content, varied between cultivars and was significantly affected by *Fusarium* inoculation (Figures 2–4). Time T1 for the cultivar samples ranged from 3.3 min (cv. ‘Sailor’) to 8.2 min (cv. ‘Tonacja’) (Table 5). However, the influence of *Fusarium* inoculation varied between cultivars. A reduction in time T1 was observed for four of the six tested cultivars. The highest difference was stated for cv. ‘Tonacja’ (on average 5 min), while for cv. ‘Pokusa’, there was no change in this parameter, and for cv. ‘Sailor’, T1 increased by 0.7 min.

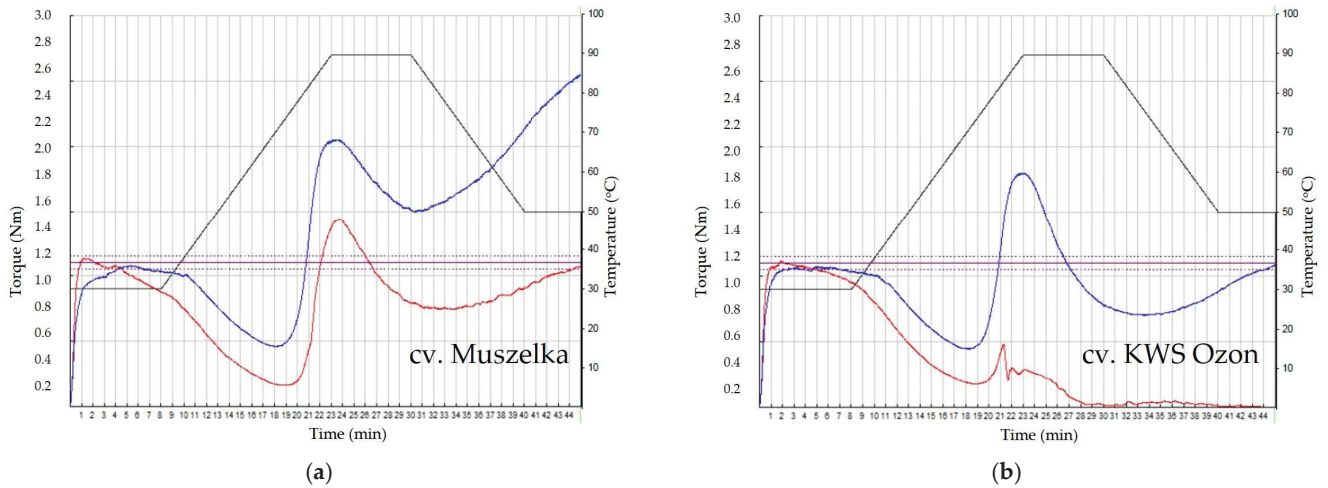


Figure 2. Comparison of Mixolab curves between control (blue lines) and *Fusarium culmorum*-inoculated (red lines) cultivars: (a) ‘Muszelka’; (b) ‘KWS Ozon’.

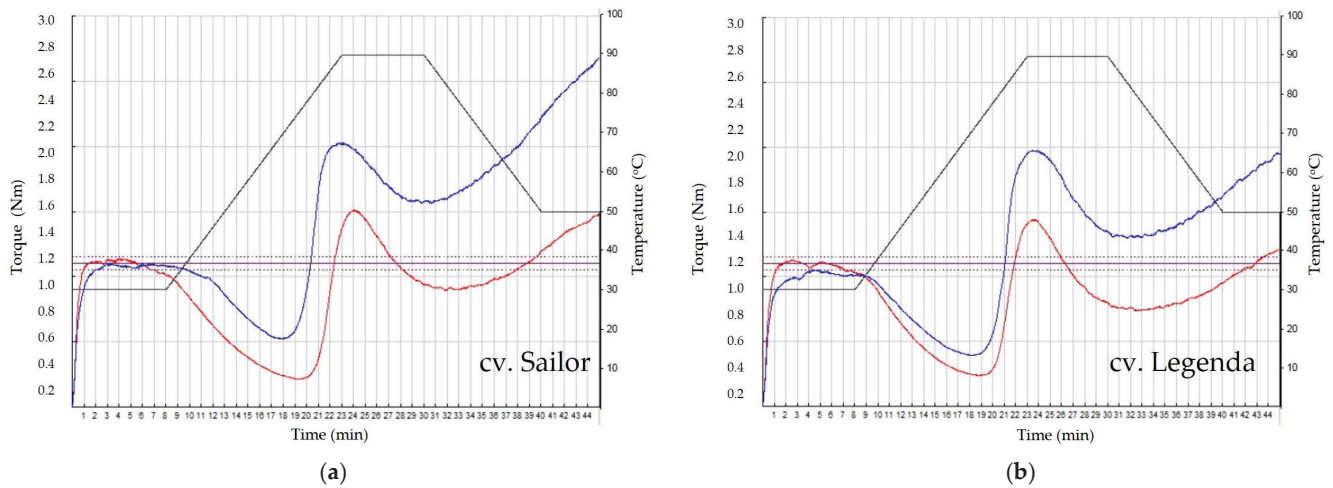


Figure 3. Comparison of Mixolab curves between the control (blue line) and *Fusarium culmorum*-inoculated (red line) cultivars: (a) ‘Sailor’; (b) ‘Legenda’.

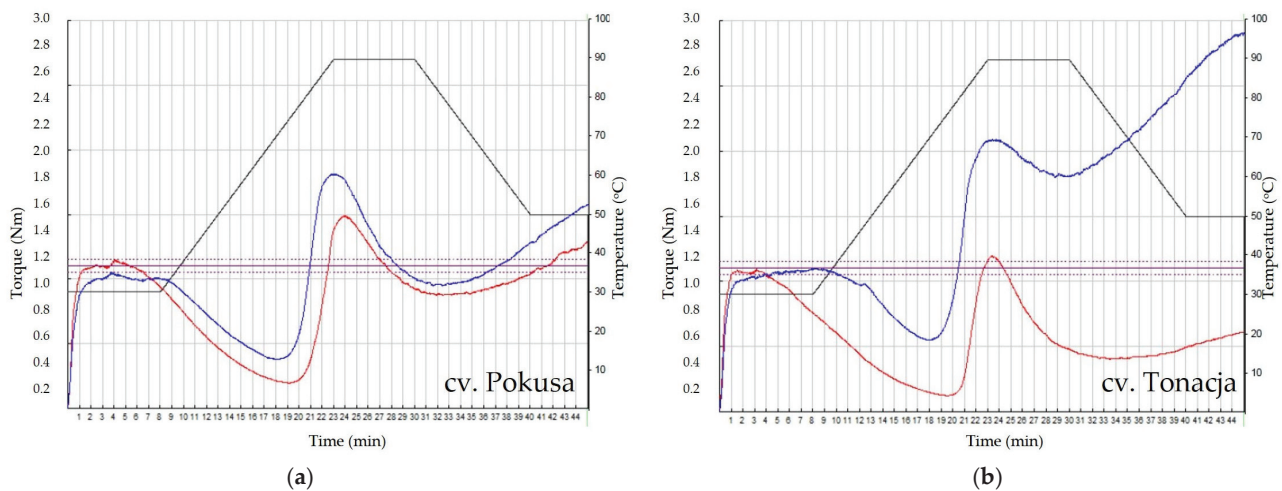


Figure 4. Comparison of Mixolab curves between control (blue line) and *Fusarium culmorum*-inoculated (red line) cultivars: (a) ‘Pokusa’; (b) ‘Tonacja’.

Dough stability, measured as the time until the upper frame of the Mixolab curve decreases by 11% from the point C1, corresponds to the dough strength. The value of this

parameter significantly depended on the experimental factors (Table 4). Among the control samples of wheat cultivars, the highest stability during dough mixing was found for the cv. ‘Tonacja’ (11.8 min), while the lowest value was observed for cv. ‘Pokusa’ (8.4 min). Inoculation with *Fusarium* fungi caused a significant decrease in dough stability for all tested cultivars, by an average reduction of 3.5 min. The greatest decrease was observed for cv. ‘Tonacja’ (6.6 min), while the lowest for cv. ‘Legenda’ (0.8 min), which was characterized by one of the lowest stability values among the control cultivars.

Protein weakening, as measured by Mixolab, was determined based on the change in dough consistency during temperature increases (C2, C1–C2, and slope α). The C2 parameter for the tested wheat flours obtained from control samples was within an appropriate level for baking, varying between cultivars. The lowest protein weakening (C2) was observed in cv. ‘Pokusa’ (0.40 Nm), while the highest was found in cv. ‘Tonacja’ (0.55 Nm). *Fusarium* inoculation caused a significant decrease in the C2 value (on average 0.30 Nm). The largest decrease was found for cv. ‘Tonacja’ (0.42 N m) and the lowest for cv. ‘Legenda’ (0.20 Nm). However, the C2 parameter for all tested samples ranged from 0.13 to 0.22 Nm, which corresponds to weak dough and potential issues during mechanical processing in dough preparation.

To better characterize the changes in dough properties, and referring to the studies [13,37,38], the differences in dough resistance measured at points C1 and C2, as well as C3 and C2, C3 and C4, and C5 and C4, were determined. The low quality of gluten proteins in samples inoculated with *Fusarium culmorum* was confirmed by increased differences in torque values between points C1–C2 (0.62 Nm for control samples vs. 0.94 for inoculated samples) (Table 4). The difference in dough resistance at points C1 and C2 (C1–C2) indicates changes in the structure of gluten proteins due to heating [13]. According to Koksel et al. [38], this corresponds to dough resistance to mixing. The greatest increase in the C1–C2 parameter was observed in cv. ‘Tonacja’ (0.42 Nm), which had the lowest C2 value. The smallest increase was stated for cv. ‘Legenda’ and ‘Pokusa’ (0.23 and 0.28 Nm, respectively). Winter wheat cultivars inoculated with *Fusarium culmorum* also exhibited a higher rate of protein weakening under heat, as measured by slope α ($-0.080 \text{ Nm min}^{-1}$ for inoculated grain samples vs. $-0.092 \text{ Nm min}^{-1}$ for control samples).

3.4. Rheological Properties of the Starch Complex of Tested Wheat Cultivars

The properties of starch and the activity of amylolytic enzymes are crucial for the dough fermentation process and for obtaining high-quality wheat bread. The following Mixolab parameters were used to determine the starch complex of wheat flour: starch gelatinization (C3), amylase activity (C4), and retrogradation (C5). Starch gelatinization speed and enzyme degradation speed are determined as slope β and slope γ , respectively.

The β index, which characterizes the increase in dough resistance due to the swelling of starch granules as the temperature rises from 30 to 90 °C, ranged from $0.587 \text{ Nm min}^{-1}$ (cv. ‘KWS Ozon’) to $0.782 \text{ Nm min}^{-1}$ (cv. ‘Muszelka’). Inoculation of ears with the fungus caused a significant decrease in this parameter in all analyzed wheat cultivars (Table 6). The greatest decrease in slope β was observed in cv. ‘KWS Ozon’ which also had the lowest value ($0.087 \text{ Nm min}^{-1}$) among the cultivars.

Table 6. The effect of *Fusarium culmorum* contamination of grain on the rheological properties of dough from winter wheat cultivars determined using Mixolab, which characterizes the properties of starch.

Parameter	Inoculation	Cultivar						Mean
		Sailor	Legenda	KWS Ozon	Pokusa	Muszelka	Tonacja	
Slope β (Nm/min)	Control	0.658 ^a	0.602 ^a	0.587 ^a	0.673 ^a	0.782 ^a	0.699 ^a	0.667 ^A
	<i>Fusarium</i>	0.583 ^b	0.563 ^b	0.087 ^b	0.419 ^b	0.518 ^b	0.602 ^b	0.462 ^B
	Average	0.621 ^B	0.583 ^C	0.337 ^E	0.546 ^D	0.650 ^A	0.651 ^A	
Slope γ (Nm/min)	Control	0.129 ^b	−0.138 ^b	−0.111 ^b	−0.111 ^b	−0.089 ^a	−0.071 ^a	−0.108 ^B
	<i>Fusarium</i>	−0.089 ^a	−0.111 ^a	−0.499 ^a	−0.069 ^a	−0.052 ^a	−0.089 ^a	−0.152 ^A
	Average	−0.109 ^B	−0.125 ^B	−0.305 ^A	−0.090 ^C	−0.071 ^C	−0.080 ^C	
C3 (Nm)	Control	2.02 ^a	1.99 ^a	1.81 ^a	1.84 ^a	2.06 ^a	2.09 ^a	1.97 ^A
	<i>Fusarium</i>	1.50 ^b	1.43 ^b	0.47 ^b	1.47 ^b	1.43 ^b	1.20 ^b	1.25 ^B
	Average	1.76 ^A	1.71 ^A	1.14 ^C	1.66 ^B	1.74 ^A	1.64 ^B	
C4 (Nm)	Control	1.56 ^a	1.320 ^a	0.72 ^a	0.97 ^a	1.50 ^a	1.79 ^a	1.31 ^A
	<i>Fusarium</i>	0.88 ^b	0.72 ^b	0.20 ^b	0.86 ^a	0.73 ^b	0.41 ^b	0.63 ^B
	Average	1.22 ^A	1.02 ^D	0.46 ^F	0.92 ^E	1.12 ^B	1.10 ^C	
C5 (Nm)	Control	2.67 ^a	1.97 ^a	1.10 ^a	1.61 ^a	2.56 ^a	2.91 ^a	2.14 ^A
	<i>Fusarium</i>	1.47 ^b	1.20 ^b	0.01 ^b	1.28 ^b	1.05 ^b	0.62 ^b	0.94 ^B
	Average	2.07 ^A	1.59 ^C	0.55 ^E	1.44 ^D	1.81 ^B	1.77 ^B	
C3–C2 (Nm)	Control	1.50 ^a	1.58 ^a	1.35 ^a	1.43 ^a	1.59 ^a	1.54 ^a	1.50 ^A
	<i>Fusarium</i>	1.29 ^b	1.20 ^b	0.30 ^a	1.29 ^a	1.28 ^b	1.07 ^b	1.07 ^B
	Average	1.39 ^A	1.39 ^A	0.82 ^B	1.36 ^A	1.43 ^A	1.30 ^A	
C3–C4 (Nm)	Control	0.46 ^b	0.68 ^a	1.09 ^a	0.87 ^a	0.56 ^b	0.29 ^b	0.66 ^A
	<i>Fusarium</i>	0.62 ^a	0.71 ^a	0.27 ^b	0.61 ^b	0.70 ^a	0.79 ^a	0.62 ^B
	Average	0.54 ^D	0.69 ^B	0.68 ^B	0.74 ^A	0.63 ^C	0.54 ^D	
C5–C4 (Nm)	Control	1.11 ^a	0.65 ^a	0.39 ^a	0.63 ^a	1.06 ^a	1.11 ^a	0.83 ^A
	<i>Fusarium</i>	0.59 ^b	0.48 ^a	−0.20 ^b	0.42 ^a	0.32 ^b	0.21 ^b	0.30 ^B
	Average	0.851 ^A	0.569 ^C	0.097 ^D	0.525 ^C	0.690 ^B	0.663 ^B	

a,b,A–F—homogeneous groups, statistically significant differences at $p = 0.05$.

The values of slope γ , which characterize the rate of enzymatic starch decomposition, ranged from $-0.111 \text{ Nm min}^{-1}$ (cv. ‘KWS Ozon’ and ‘Pokusa’) to $-0.071 \text{ Nm min}^{-1}$ (cv. ‘Tonacja’). Inoculation with *Fusarium culmorum* significantly affected the values of slope γ in four cultivars, causing an increase in this parameter. However, for the cv. ‘KWS Ozon’ and cv. ‘Tonacja’, the value of slope γ decreased by -0.388 and $-0.018 \text{ Nm min}^{-1}$, respectively (Table 6).

Regarding starch gelatinization (C3), control wheat flours exhibited medium to low α -amylase activity, with values ranging from 1.81 Nm (cv. ‘KWS Ozon’) to 2.09 Nm (cv. ‘Tonacja’). *Fusarium culmorum* inoculation caused significant reductions in C3 values (from 1.92 Nm for control samples to 1.25 Nm for inoculated samples). The greatest reduction was stated for cv. ‘KWS Ozon’ (-1.34 Nm to 0.47 Nm) (Figure 2) compared to other cultivars, where the reduction was on average -0.6 Nm . The C3 value for cv. ‘KWS Ozon’, with the lowest C3 in inoculated samples, was characterized by flour from sprouted grain with very high α -amylase activity.

The control winter wheat samples were more variable in terms of amylase activity (C4) and retrogradation (C5) than starch gelatinization (C3). C4 values for control samples ranged from 0.72 Nm (cv. ‘KWS Ozon’) to 1.79 Nm (cv. ‘Tonacja’) (Table 6). *Fusarium* inoculation caused a significant decrease in amylase activity (C4) (from 1.31 Nm in control samples to 0.63 Nm in inoculated samples). The highest drop was found for cv. ‘Tonacja’ (1.79 Nm to 0.41 Nm), and the lowest for cv. ‘Pokusa’ (0.97 Nm to 0.86 Nm). The

lowest value of C4 was stated for cv. 'KWS Ozon' (0.20 Nm), confirming the stickiness of the dough.

Starch retrogradation in the cooling stage of Mixolab analysis varied between control winter wheat samples. The highest C5 value was recorded for cv. 'Tonacja' (2.91 Nm), compared to the very low value for cv. 'KWS Ozon' (1.10 Nm). Similar to other starch complex characteristics, starch retrogradation was strongly influenced by *Fusarium culmorum* inoculation (on average 2.14 vs. 0.94 Nm, respectively). The largest difference was found for cv. 'Tonacja' (a drop of 2.29 Nm) and the smallest for cv. 'Pokusa' (a drop of 0.33 Nm).

In partial agreement with the previous evaluation of the Mixolab curve, the differences between C3–C2, C3–C4, and C5–C4 were also analyzed. Dough properties related to starch gelatinization are determined at point C3 of the graph, and the difference in dough resistance at points C3 and C2 (C3–C2) are determined [39]. The value of the C3–C2 parameter was influenced by the cultivar and *Fusarium* inoculation. Inoculation caused a significant decrease in the value of this parameter. Flour from control cv. 'Muszelka' exhibited the highest C3–C2 value (1.59 Nm), while cv. 'KWS Ozon' showed the lowest (1.35 Nm). The greatest decrease in C3–C2 was observed in cv. 'KWS Ozon' (a drop of 1.05 Nm), whereas the reduction for inoculated cv. 'Pokusa' was only 0.14 Nm (Table 6).

Control samples of cv. 'KWS Ozon' were characterized by the highest differences between C3–C4 (1.09 Nm), indicating the highest α -amylase activity or less resistance to shear thinning. In contrast, cv. 'Tonacja' had the lowest C3–C4 difference (0.29 Nm), corresponding to low α -amylase activity. *Fusarium* inoculation caused an increase in C3–C4 difference in four of the six cultivars. In the case of cv. 'KWS Ozon', the dough became sticky at points C3 and C4, making it difficult to analyze, which explains the opposite change in this parameter.

The difference in dough resistance between points C5 and C4 (C5–C4) reflects the susceptibility of bread to staling and may indicate its shelf life, as described by Papoušková et al. [13]. The smallest difference was observed in the flour of cv. 'Pokusa' and the largest in cv. 'Tonacja' and cv. 'Sailor'. *Fusarium* inoculation significantly affected the values of this parameter (Table 6). Among the cultivars after inoculation, cv. 'KWS Ozon' was assessed as the most favorable, as it exhibited the lowest C5–C4 value, suggesting the potential for the longest shelf life of the obtained bread. On the other hand, cv. 'Sailor' and cv. 'Tonacja' were considered the least favorable. For cv. 'KWS Ozon', negative values of the C5–C4 parameter in the flour after grain inoculation indicated very high amylolytic activity and technological problems in bread production.

3.5. Mixolab Wheat Flour Profiles

The Mixolab profiler allows the evaluation of dough consistency over time and determines, in the same assay, both the mixing and pasting characteristics of flour during a steady increase in temperature [40]. The Mixolab profiler provides users with the ability to classify the quality of tested flours based on six quality criteria: water absorption, mixing, gluten+, viscosity, amylase, and retrogradation, with values ranging from 0 to 9. This quality control tool, based on both protein and starch characteristics, helps to better screen and detect differences between tested samples and identify underperforming flours.

The Mixolab profilers obtained in our research significantly differed between the control cultivar samples and those inoculated with *Fusarium culmorum* (Figures 5–7). Differences were also noted between the tested cultivars.

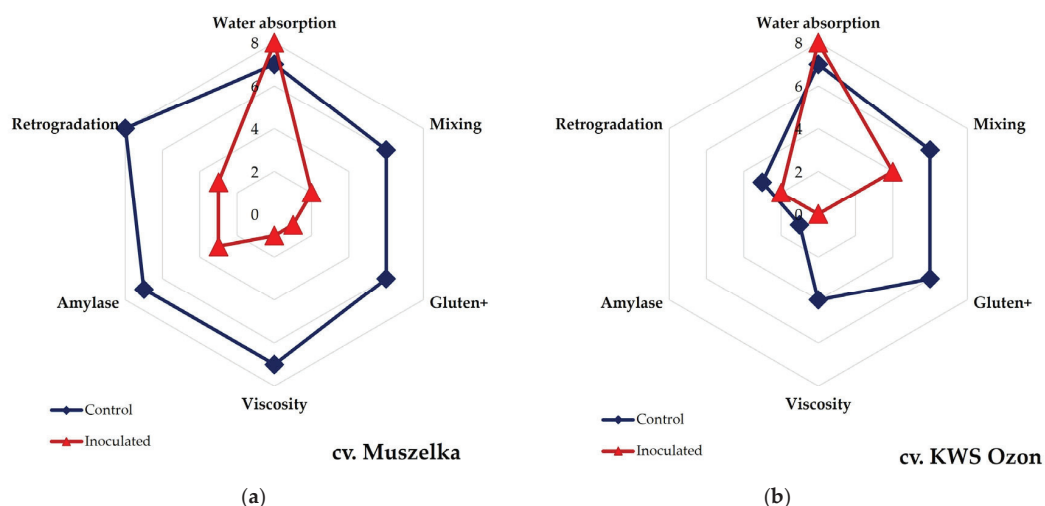


Figure 5. Mixolab profiler values of whole-grain wheat flours obtained from: (a) cv. ‘Muszelka’; (b) cv. ‘KWS Ozon’ from both control samples and samples inoculated by *Fusarium culmorum*.

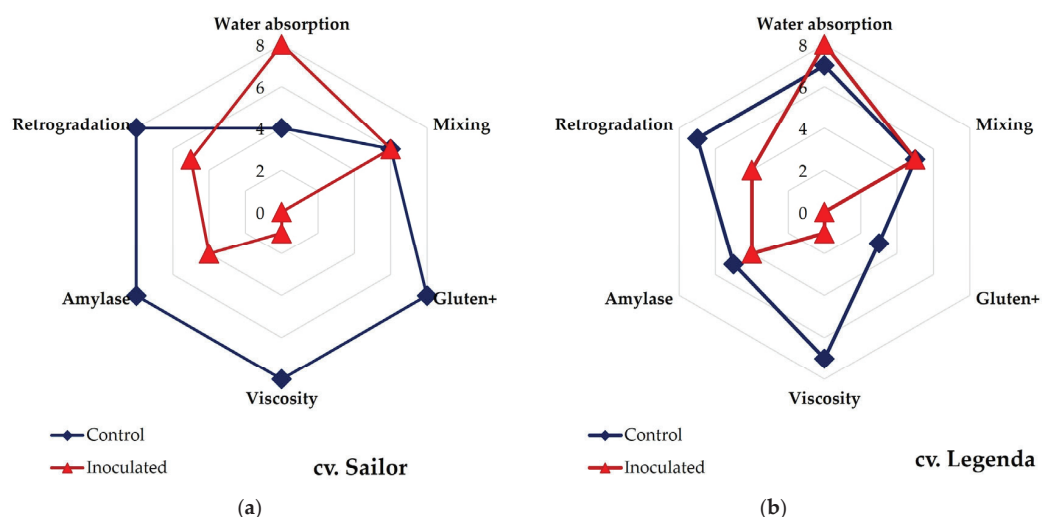


Figure 6. Mixolab profiler values of whole-grain wheat flours obtained from: (a) cv. ‘Sailor’; (b) cv. ‘Legenda’ from both control samples and samples inoculated by *Fusarium culmorum*.

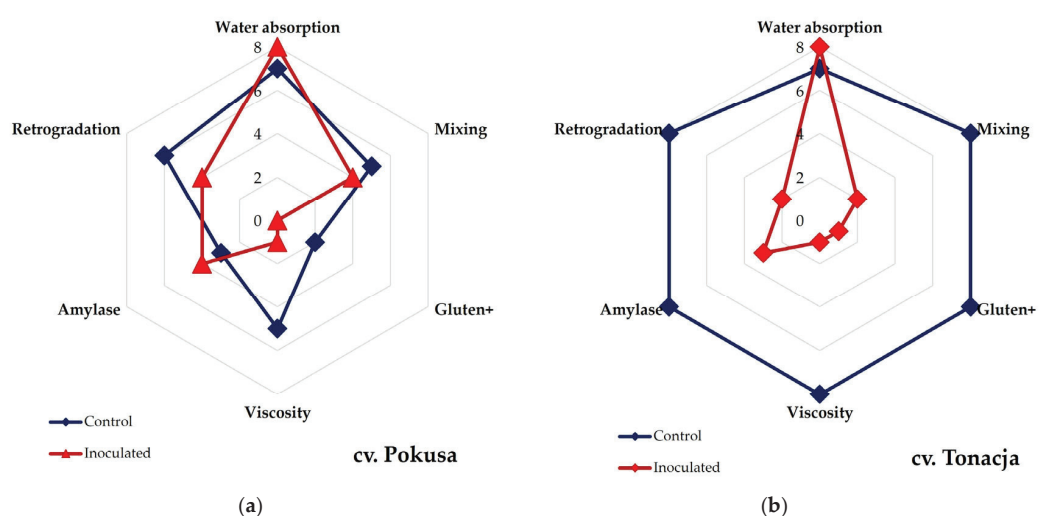


Figure 7. Mixolab profiler values of whole-grain wheat flours obtained from: (a) cv. ‘Pokusa’; (b) cv. ‘Tonacja’ from both control samples and samples inoculated by *Fusarium culmorum*.

The water absorption index, which corresponds to the ability of flour to absorb water, was in the range of 4 (cv. 'Sailor') to 7 (other cultivars) among the control samples. *Fusarium* inoculation significantly increased the water absorption index to 8 in each cultivar.

In contrast, other parameters showed the opposite effect. The mixing index, which corresponds to the stability of flour dough during kneading, is higher for stronger dough. Control samples of wheat cultivars had a mixing index ranging from 5 (medium quality—cv. 'Legenda', cv. 'Pokusa') to 8 (strong gluten structure—cv. 'Tonacja'). *Fusarium* inoculation weakened the gluten structure, lowering the mixing index (an average reduction of 2 units). However, different effects were observed across cultivars. The greatest reduction in this parameter was seen in cv. 'Tonacja' (Figure 7), while cv. 'Sailor' and cv. 'Legenda' showed no effect (Figure 6).

The gluten+ index describes the resistance of gluten to heat and the changes in the quality of gluten proteins under the influence of an increase in temperature from 30 °C to 60 °C. In non-inoculated samples, this index ranged from 2 to 8, with the highest value observed in the control samples of cv. 'Sailor' and cv. 'Tonacja' and the lowest in cv. 'Pokusa'. *Fusarium* inoculation significantly reduced the heat-induced weakening of gluten proteins, lowering the gluten+ index by an average of 5 units to 0 (cv. 'Sailor', 'Legenda', 'KWS Ozon', 'Pokusa') and 1 (cv. 'Muszelka' and cv. 'Tonacja'), indicating a reduction in stability to heat.

The next index with significant differences observed was viscosity, which corresponds to changes in dough characteristics as the temperature increases from 60 °C to 80 °C. This index is influenced by both amylase activity and starch quality, ranging from 4 (cv. 'KWS Ozon') to 8 (cv. 'Sailor' and cv. 'Tonacja'). *Fusarium* inoculation significantly lowered the viscosity index to 0 (cv. 'KWS Ozon') and 1 (the rest of the cultivars). The average drop of 6 units was the highest among the other Mixolab Profiler indexes.

The amylase index, reflecting changes in dough properties due to the α -amylase enzyme, ranged from 1 (cv. 'KWS Ozon') to 8 (cv. 'Sailor' and cv. 'Tonacja'). *Fusarium* inoculation caused a significant decrease in this index in cv. 'Muszelka' (Figure 5), cv. 'Sailor' (Figure 6), and cv. 'Tonacja' (Figure 7) compared to the control samples. However, an increase in the viscosity index was observed in cultivars with the lowest amylase index in the control samples: cv. 'KWS Ozon' (from 1 to 4) and cv. 'Pokusa' (from 3 to 4).

The retrogradation index, which depends on the characteristics of the starch and its hydrolysis during the test, ranged from 3 (cv. 'KWS Ozon') to 8 (cv. 'Sailor', cv. 'Muszelka', cv. 'Tonacja') in non-inoculated cultivars. In flour samples from grain contaminated with *Fusarium culmorum*, a significant reduction in this index was observed (on average a drop of 3 units).

3.6. Principal Component Analyses (PCAs)

Almost all investigated factors were presented in a two-dimensional coordinate system (Figure 8). Based on the results, we identified four distinct clusters of samples. The first group consisted of KWSOI (blue color), the second group included TC, MC, and SC (red color), the third group comprised PI, MI, SI, TI, and LI (green color), and the fourth group included LC, KWSOC, and PC (black color). Notably, opposite properties were observed between the first and fourth groups of samples. A similar trend was noted between the second and third clusters. The KWSOI sample was characterized by a high ash content and water absorption. In contrast, the LC, KWSOC, and PC samples exhibited higher rheological parameters (C3 and C3–C2), sedimentation index, and gluten content. The samples PI, MI, SI, TI, and LI stood out due to their total protein content and specific rheological parameters (C1–C2 and C3–C4). On the other hand, the TC, MC, and SC samples were distinguished by TKW, sedimentation time, and other rheological parameters (T1, C2, C4, C5, and C5–C4).

It is worth noting that all inoculated samples were positioned on the left side of the score plot, while the control samples were located on the right side. This distribution indicates a negative interdependence between the different plant treatments.

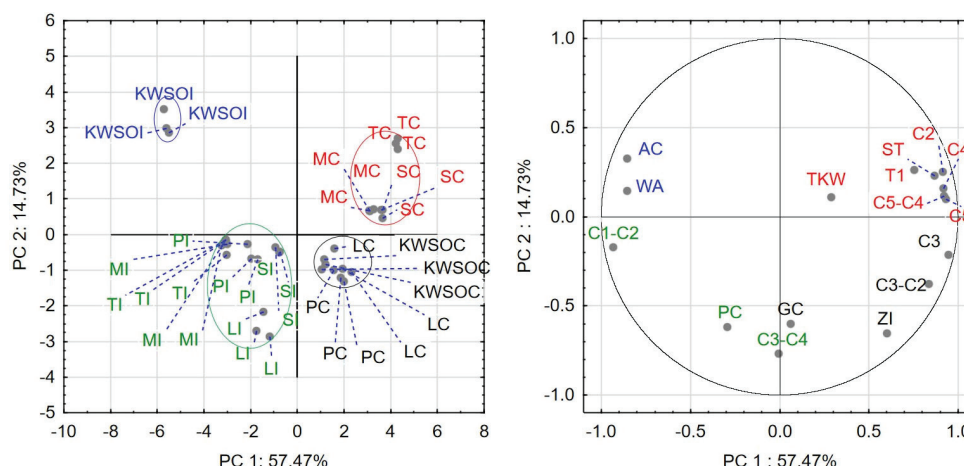


Figure 8. Principal component analyses represented by two-dimensional plot: score plot (left side) and loading plot (right side). Explanation for cases: KWSOI—KWS Ozon Inoculated; KWSOC—KWS Ozon Control; TI—Tonacja Inoculated; TC—Tonacja Control; MI—Muszelka Inoculated; MC—Muszelka Control; SI—Sailor Inoculated; SC—Sailor Control; PI—Pokusa Inoculated; PC—Pokusa Control; LI—Legenda Inoculated; LC—Legenda Control. Explanation for variables: TKW—Thousand kernel weight; PC—Protein content; GC—Gluten content; ZI—Zeleny sedimentation index; AC—Ash content; T1—Time T1 (rheological parameter); ST—Stabilization time (rheological parameter); WA—Water absorption; C2, C3, C4, C5, C1–C2, C3–C2, C3–C4, C5–C4—Rheological parameters of dough determined by Mixolab equipment.

4. Discussion

The literature review and the results of our studies indicate that *Fusarium culmorum* is pathogenic to winter wheat and produces the mycotoxin deoxynivalenol (DON) in the grain [21,41,42]. The results indicate differences in the response of wheat cultivars to stress caused by *Fusarium culmorum* inoculation. This is confirmed by changes in the properties of the starch and protein complex induced by *Fusarium culmorum* inoculation. The research hypothesis was tested through conducting a field experiment and laboratory tests. They included the inoculation of *Fusarium culmorum*, which, according to the literature, is the species that most commonly infects wheat in Polish weather conditions and produces the largest amounts of mycotoxins [29,43].

4.1. Mycotoxin Content in Grain of Winter Wheat Cultivars

The presented studies focused on the inoculation of *Fusarium culmorum* as it is a predominant species in Polish conditions and produces the most mycotoxins [29,44,45]. Bryła et al. [44] examined the levels of 26 mycotoxins in cereal grains in Poland and found the presence of DON in all tested wheat samples. The studies described different levels of DON depending on the crop year. For the infection of wheat by *Fusarium ssp.*, the most crucial factors are the weather conditions during the flowering period and the initial ripeness of wheat (May and June). As shown in Table 2, the weather conditions in 2019 were not favorable for the development of *Fusarium ssp.* The air temperature was higher in May and June, by 1.8 and 3.3 °C, respectively, compared to 2020. There were very large differences in the amount of precipitation—2019 was a very dry year. The amount of rainfall in 2020 in May and June was 26.6 mm and 150.8 mm higher, respectively, than in 2019. During the flowering period, it was too dry for the fungi to develop in the wheat

grains even after artificial inoculation. Weather conditions caused the DON level to be very low in 2019, therefore only seeds from 2020 were used in our experiment.

The studies found different levels of DON depending on the cultivars. The lowest content of this mycotoxin was found in cv. ‘Sailor’, while the highest was in cv. ‘Muszelka’ and cv. ‘Legenda’. Selection of genotypes resistant to *Fusarium* head blight (FHB) is one of the methods used in wheat cultivation to reduce the occurrence of mycotoxins in wheat grain. Resistance to FHB is controlled by major and minor genes located on all wheat chromosomes, except for chromosome 7D [46]. The presented research results confirm the literature data indicating differences in the amounts of *Fusarium* mycotoxins in individual wheat cultivars grown under the same weather conditions [29,42,44]. The differences in the amount of mycotoxins in wheat grain may result from different types of resistance [47–49]. Therefore, there is not always a highly significant relationship between resistance to the *Fusarium* disease complex and the accumulation of mycotoxins. This was confirmed in our studies, as it was shown that cultivars with high resistance to FHB (cv. ‘Legenda’) had higher levels of mycotoxins than cultivars characterized by lower resistance (cv. ‘Sailor’). Most likely, these cultivars are characterized by resistance to the accumulation of *Fusarium* toxins in the grain. Studies by Siou et al. [50] report that the cropping of cultivars with a short vegetation period or early flowering allows for the avoidance or significant reduction of infection with fungi of the genus *Fusarium* and the accumulation of toxins. This relationship was not confirmed in the presented studies. It may have resulted from the fact that the difference in the earliness of cultivars was only 5 days. Another factor that is most likely the reason for the varying amounts of toxin accumulation is the structure and composition of grain. Walter et al. show that cell walls and the aleurone layer are significant physical barriers to the penetration of the fungus [51]. Literature data also indicate that other morphological features such as grain color, hulls, wax coating, and ferulic acid concentration affect the levels of mycotoxins in wheat grain [42].

The wheat microbiome plays a crucial role in limiting infections caused by *Fusarium* spp., which are responsible for diseases such as *Fusarium* head blight and root rot. The plant microbiota can influence plant health through various mechanisms, including competition with pathogens, induction of plant immunity, and the production of antimicrobial substances. Microorganisms in the wheat microbiome, particularly bacteria and fungi, can produce a range of antimicrobial compounds that inhibit pathogen growth. Some *Bacillus* strains produce lipopeptides that act as antimicrobial agents, inhibiting the development of *Fusarium* [52]. Other bacteria produce enzymes, such as chitinases, which can degrade the cell walls of *Fusarium* fungi, limiting their growth and increasing biodiversity within the microbiome. The biodiversity of the wheat microbiome may also play a key role in protecting against *Fusarium* infections. Studies have shown that plant microbiomes with higher biodiversity can improve plant health and enhance resistance to diseases caused by *Fusarium* [53,54]. The biodiversity of the wheat microbiome may also play a key role in protecting against *Fusarium* infections. Diverse microbial communities in the rhizosphere can collaborate to more effectively suppress pathogens.

4.2. Influence of *Fusarium culmorum* Infection on the Grain Quality and Rheological Properties of Dough

The technological value of wheat depends on the basic quality indicators, including, among others, the total protein content and its quality, the quantity and quality of gluten, the Zeleny sedimentation index, and the degree of starch damage [7,55]. To assess wheat flour quality more precisely, tests can be extended to include the rheological properties of the dough, which are performed using devices such as the farinograph, extensograph, and alveograph. Based on the data obtained from these tests, changes in the dough’s consistency and extensibility can be assessed. The viscosity of the flour suspension in water

during heating is determined using an amylograph [56]. A comprehensive assessment of rheological properties requires the use of several devices. In the studies discussed, Mixolab was used to assess the quality of whole-grain wheat flour. This device allows for the simultaneous evaluation of both protein and starch properties [40,56].

The impact of wheat infection by fungi of the *Fusarium* genus and grain contamination with mycotoxins on technological value has been analyzed in only a few studies. Typically, the effect of *Fusarium* on individual quality features, such as nutrient content, protein content, gluten quantity and quality, Zeleny sedimentation index, or dough rheological properties, has been taken into account [21,57,58]. According to the literature, *Fusarium* infection of grains causes changes in the proportion of seed coat to endosperm, which results in an increase in ash content [11–13]. These relationships were confirmed in the present study and were visualized in PCA charts. In all cultivars, inoculation with *Fusarium culmorum* led to an increase in ash content. The highest increase was recorded in the cv. ‘Pokusa’ (0.44%), while the lowest was in the cv. ‘Tonacja’ (0.13%) and cv. ‘Legenda’ (0.11%).

Another aspect of the study was to identify wheat genotypes less susceptible to the reduction of thousand kernel weight (TKW) due to *Fusarium* infection. This is of significant importance for both yield size and quality, as TKW affects the milling and baking value of flour [15]. Cultivars with low TKW are characterized by low flour extraction due to disturbed proportions of endosperm to fruit-seed coat. While they have higher protein content, these proteins are not accumulated in the endosperm but in the fruit-seed coat, which is irrelevant for flour production. In the present study, the greatest reduction in TKW due to *Fusarium* inoculation was observed in the cv. ‘Legenda’, ‘Muszelka’, and ‘Tonacja’, which was proved in PCA charts. The results of these studies are consistent with those of Packa et al. [59], who found a reduction in TKW under *Fusarium* infection, with an average of 13% over several years.

Proteins stored in wheat grain are an important source of nutrients for humans and play a major role in determining bread baking quality [7]. The present research showed an increase in protein content after *Fusarium culmorum* inoculation, with an average increase of 1.1% percentage point across the cultivars (PCA chart). The highest increase, 3.5% compared to the control, was observed in the cv. ‘Legenda’, while the lowest increases were seen in the ‘Pokusa’ and ‘Muszelka’ cultivars. In the cv. ‘KWS Ozon’, the difference was not significant. The results suggest a significant change in one of the most important quality indicators [7]. These findings align with the literature, which indicates an increase in protein content due to the *Fusarium* contamination, as well as showing varietal differences [14,25]. The increase in protein content in the grain may be a response to stress, with plant cells synthesizing specific proteins and potentially altering their fractional and amino acid composition [60,61]. These proteins help mitigate the effects of stress by neutralizing its impact and protecting essential cellular structures and metabolic processes [62].

From the perspective of flour suitability for baking, both the quantity and quality of protein are important. The distinguishing factor determining both values is the sedimentation index. In the present studies, the sedimentation index was found to decrease by an average of 10.7% in cultivars subjected to *Fusarium* inoculation (PCA chart). Similar results were obtained by El Chami et al. [21]. Research by Gärtner et al. indicates varietal differences in this index, a finding confirmed in this work [14]. Kreuzberger et al. and Wang et al. obtained different results, suggesting no effect of *Fusarium* mycotoxins on the sedimentation index [17,18]. This discrepancy may be attributed to the selection of cultivars.

Based on the results, cultivars were classified into those where the sedimentation index was significantly reduced (cv. ‘KW Ozon’, cv. ‘Pokusa’), those with an increase (cv. ‘Legenda’), and those that showed no effect of *Fusarium* on the sedimentation index

(cv. ‘Muszelka’ and cv. ‘Tonacja’). Changes in the sedimentation index reflect the impact of *Fusarium* on the balance between gliadins and glutenins that form gluten. Therefore, it can be concluded that, among the cultivars compared, ‘Muszelka’ and ‘Tonacja’ were more resistant to changes in protein quality due to *Fusarium* infection, as evidenced by the negligible change in gluten content in these cultivars.

In addition to determining the effect of *Fusarium culmorum* inoculation on the qualitative characteristics of grain, the effect on the quality of starch–protein complex was also examined. Laboratory tests were performed using Mixolab, which allowed for a comprehensive assessment of the experimental factors’ impact on the baking value and dough quality. It is worth noting that the quality was assessed based on the quality parameters of the starch and protein complex.

According to Gärtner et al., flour obtained from wheat cultivars containing mycotoxins, compared to flours from uncontaminated cultivars, is characterized by reduced baking quality parameters such as water absorption and dough softening [14]. Similar results were obtained by Horvat et al., who compared the rheological properties of dough from contaminated and uncontaminated flours [26]. They found a reduction in water absorption by 1.6%, dough development time by 17%, and quality number by 19.9%. The authors also showed that *Fusarium* contamination reduced dough energy, maximum tensile strength, and the strength-to-extensibility ratio by 57.7%. Additionally, they observed a negative effect on the specific volume of bread. Similarly, the presented studies demonstrated a negative effect of *Fusarium* on flour and dough properties. Water absorption of flours from inoculated cultivars increased by an average of 3.6% compared to uninfected grain, and dough development time was shortened by an average of 44.5%. A decrease in dough stability was also observed, with an average reduction of 34.4%, alongside changes in dough resistance to kneading depending on the cultivars. These changes were likely caused by proteolytic enzymes of *Fusarium*, which remain inactive in the grain but can be activated during dough kneading [18,19]. In the research by Peršić et al. [63], FHB infection did not affect the water absorption of wheat flour from either location. However, FHB infection caused the most significant increase in dough softening, by over 400% compared to control samples in one of the locations, suggesting significant genotypic variability.

The results also indicate a negative effect of *Fusarium* on starch properties, particularly the swelling of starch granules and the rate of enzymatic degradation. Changes in starch properties under the influence of *Fusarium* have been confirmed in the literature [20–22]. The presented study found that the effect of *Fusarium* on starch degradation varied depending on the genotype. The β index, which characterizes the increase in dough resistance due to starch granule swelling, decreased significantly in all analyzed wheat cultivars. In contrast, the γ index, indicating the high amylolytic enzyme activity, varied between the cultivars. The greatest decrease in the γ index, indicating high amylolytic enzyme activity, was found in cv. ‘KWS Ozon’, the cultivar with the highest cumulative level of DON. As a result, the β index, indicating a reduction in dough resistance, was also reduced. Similar conclusions were drawn by Gärtner et al. and Wang et al. [14,18].

5. Conclusions

The effect of *Fusarium culmorum* inoculation on the grain quality, starch properties, and protein complex was investigated, resulting in changes in flour properties and dough rheological characteristics. *Fusarium culmorum* inoculation led to an increase in protein, gluten, and ash content, a decrease in the sedimentation index, an increase in flour water absorption, as well as changes in dough rheological properties: a shorter dough development time, reduced dough stability time, decreased dough resistance to mixing, and reduced dough consistency. Inoculation caused changes in starch properties, as indicated

by the determination of the β slope parameter in Mixolab (the rate of enzymatic starch decomposition) and the γ slope (starch gelatinization). It also led to a weakening of dough consistency. The observed interaction between *Fusarium culmorum* inoculation and the wheat cultivars in shaping the qualitative parameters and rheological properties of the dough indicates that some wheat cultivars include less susceptible genotypes, which do not exhibit any changes as a result of *Fusarium* infection.

Analyses of the technological value of grain showed that there were no significant differences between cv. 'KWS Ozon', cv. 'Tonacja', and cv. 'Muszelka' in terms of protein content, gluten content, and Zeleny sedimentation index. It was shown that, in the case of 'Pokusa' cv., the effect of *Fusarium* inoculation was not significant in contrast to 'KWS Ozon' cv., where a significant effect on starch–protein complex was observed.

The studies have shown that, under the same environmental and cultivation conditions, different cultivars accumulate various amounts of mycotoxins. From a scientific perspective, it is important to further investigate the underlying causes of this variability. The 'Pokusa' and 'Sailor' cultivars may serve as a valuable source of resistance to mycotoxin accumulation in grain, as they exhibited the lowest contamination with DON among all tested cultivars.

A review of the literature suggests that the cultivar's microbiome may play a key role in this process, therefore, future research should consider this aspect.

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Article

Foliar Spraying with Endophytic *Trichoderma* Biostimulant Increases Drought Resilience of Maize and Sunflower

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Abstract: Microbial biostimulants that promote plant growth and abiotic stress tolerance are promising alternatives to chemical fertilizers and pesticides. Although *Trichoderma* fungi are known bio-control agents, their biostimulatory potential has been scarcely studied in field conditions. Here, the mixture of two endophytic *Trichoderma* strains (*Trichoderma afroharzianum* TR04 and *Trichoderma simmonsii* TR05) was tested as biostimulant in the form of foliar spray on young (BBCH 15–16) maize (5.7 ha) and sunflower (5.7 and 11.3 ha) fields in Hungary. The stimulatory effect was characterized by changes in plant height, the number of viable leaves, and the chlorophyll content, combined with yield sensor collected harvest data. In all trials, the foliar treatment with *Trichoderma* spores increased photosynthetic potential: the number of viable leaves increased by up to 6.7% and the SPAD index by up to 19.1% relative to the control. In extreme drought conditions, maize yield was doubled (from 0.587 to 1.62 t/ha, $p < 0.001$). The moisture content of the harvested seeds, as well as sunflower height, consistently increased post-treatment. We concluded that foliar spraying of young plants with well-selected endophytic *Trichoderma* strains can stimulate growth, photosynthesis, and drought tolerance in both monocot maize and dicots sunflower crops in field conditions.

Keywords: *Trichoderma afroharzianum*; *Trichoderma simmonsii*; drought tolerance; chlorophyll content; maize yield; fungal biostimulant; foliar spray

1. Introduction

The overuse of synthetic chemical fertilizers and pesticides poses significant environmental challenges, contributing to biodiversity loss and raising concerns about soil health, plant integrity, and food safety [1–4]. Additionally, climate change—characterized by severe droughts, storms, and declining biodiversity—further exacerbates these issues by reducing crop yields, diminishing soil fertility, and increasing the prevalence of pests and disease outbreaks [5–8]. In response to these challenges, sustainable crop production must strike a delicate balance by minimizing reliance on chemical fertilizers and pesticides while ensuring stable yields under increasingly adverse environmental conditions [7].

Beneficial microorganisms offer a promising solution by promoting plant growth and alleviating environmental stresses through diverse mechanisms. These microorganisms enhance nutrient uptake and improve the solubilization of phosphorus, potassium, iron, and

zinc by producing organic acids and siderophores. Additionally, they help mitigate biotic and abiotic stresses by synthesizing phytohormones, inducing systemic resistance, boosting the activity of defense-related enzymes, and increasing the accumulation of osmotically active substances in plants [9–13]. Furthermore, these fungi can mitigate abiotic stress by activating endogenous plant defense responses and altering plant metabolism [14–16].

Among these microorganisms, *Trichoderma* spp. stand out as versatile fungi capable of functioning as biocontrol agents, plant growth stimulators, and biofertilizers [17–19]. These fungi enhance nutrient availability by producing organic acids and siderophores, which foster robust root development [11]. Furthermore, *Trichoderma* spp. directly promote plant growth through phytohormone production [10,20] and mitigate abiotic stresses by activating endogenous plant defenses and modifying plant metabolism [14–16].

The biocontrol and biostimulant properties of *Trichoderma* spp. have been investigated across various crops, including maize and sunflower, through soil or seed treatments [21–25]. While most research has been conducted in controlled environments, further field studies are necessary to validate these findings and assess the long-term impacts of *Trichoderma* applications. This aspect is particularly significant, as *Trichoderma* spp. predominantly colonize root tissues; however, endophytic strains have also been identified, and their biocontrol potential in annual crops remains to be explored [26,27].

In this study, we evaluated the effects of a commercially formulated endophytic *Trichoderma* biostimulant, applied as a foliar spray, on maize and sunflower under field conditions in Hungary. The product, containing two endophytic strains—*Trichoderma afroharzianum* TR04 and *Trichoderma simmonsii* TR05—was derived from woody grapevine tissues [26]. Our results demonstrate the biostimulant efficacy of this formulation in enhancing crop performance in these key monocot and dicot crops in practice under field conditions.

2. Materials and Methods

2.1. Experimental Sites and Treatments

These experiments were conducted with maize in 2022 at Experimental Site I, with sunflower in 2022 at Experimental Site II, and in 2023, at Experimental Site I. Both experimental sites are located on the outskirts of Hajdúszovát, Hungary, in the South-Hajdúság meso-region of the Great Hungarian Plain.

The soil at both sites is loamy or clay-loam meadow chernozem with a humus content of 3.2–3.5% and a pH of 6.7–7.15 (determined by accredited laboratory). The average altitude of the sites is 92 m above sea level. The GPS coordinates for the center of Experimental Site I (Figure 1a) are 47°23'36" N 21°24'50" E, and for Experimental Site II (Figure 1b), they are 47°22'16" N 21°26'12" E. In all cases, the field was planted with maize the previous year.

The control and the *Trichoderma*-treated plots were set up in four alternating strips (Figure 1). Sunflower NK Neoma (early) hybrid was seeded on the 21st and 22nd April 2022 and 2023. Maize Dekalb DKC4897 (FAO 390–400) hybrid was also seeded on 21 April 2022. Monosem NG Plus 6 (Monosem Inc., Edwardsville, KS, USA) sowing machine was used in all cases. Plant density was 60,500 plants per hectare for sunflower and 74,000 plants per hectare for maize. All experiments were treated one week after applying post-emergent weed control at the BBCH 15–16 phenological stages of the plants [28]. This phase provides sufficient plant surface for effective colonization by the endophytic strains and allows for combination with herbicide treatment. The application was carried out using a John Deere 6120M tractor equipped with AutoTrac and a StarFire 6000 antenna (John Deere Inc., Mannheim, Germany). The navigation system was utilized, with the machine's working width set and tracks recorded for accurate operation. Control plots were sprayed with water in amounts equivalent to the water content of the *Trichoderma* formulation. *Trichoderma*-treated plots were sprayed with a freshly prepared solution of Tricho Immun (Danuba, Szentendre, Hungary), which contained a mixture of *Trichoderma afroharzianum* TR04 and *Trichoderma simmonsii* TR05 conidia (2×10^8 CFU/g) on a substrate consisting of glucose and perlite powder. The viability of the conidia was previously tested in the laboratory. The

application rate was 1 kg/ha in 300 L of water per hectare, following the manufacturer's protocol [29]. The plot width was 18 m, corresponding to the working width of the Kertitox Revolution (Farmgép, Debrecen, Hungary) trailed field sprayer, which is three times the 6 m working width of the combine harvester (Figure 1c).

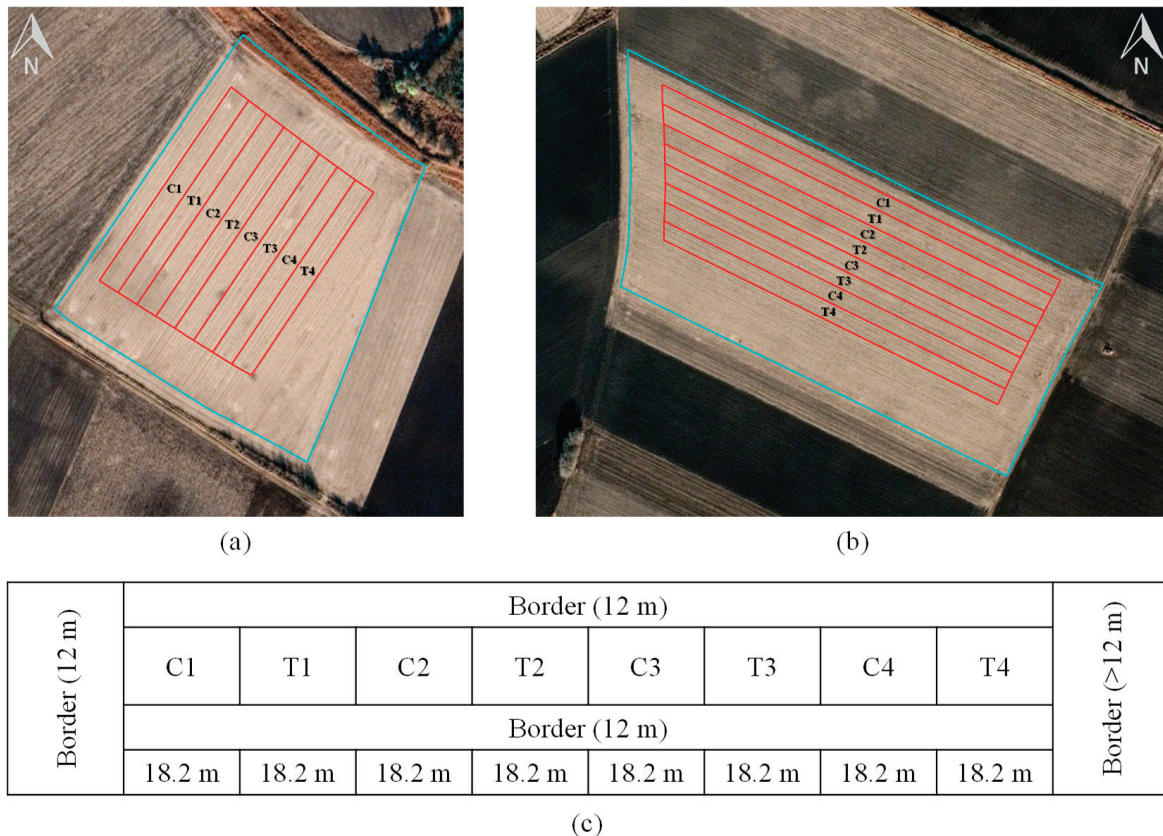


Figure 1. Experimental design to study the effect of the endophytic *Trichoderma* formulation. Aerial view of Experimental Site 1 (a) and Experimental Site 2 (b). Blue lines indicate the borders of the Experimental Sites, red lines indicate the borders of the plots. (c) Experimental designs C1–4 indicate control plots; T1–4 indicate plots treated with *Trichoderma*.

Meteorological data were collected with a Campbell Scientific (Campbell Scientific Ltd., Logan, UT, USA) meteorological station with Campbell HygroVUE10 temperature and humidity sensor, Kipp&Zonen CMP-11 pyranometer, and PG-200 weighing rain gauge. It is operated by the Centre for Precision Farming R&D Services, FAFSEM, University of Debrecen. The weather station is located in the Agrometeorological Observatory, Debrecen-Kismacs, approximately 20 km from the experimental sites. The annual meteorological diagram was created using monthly resolution data, while the differences during the vegetation period are based on weekly resolution data.

2.2. Evaluation of Experimental Site Homogeneity Based on NDVI

Normalized Differential Vegetation Index (NDVI) was calculated using data from Sentinel-2 satellites (launched by the Global Environment and Security Monitoring Program of the European Space Agency). The calculation was based on red and infrared spectral values from 10 m spatial grids, as described by Rouse et al. (1974) [30]:

$$NDVI = \frac{\lambda_{NIR} - \lambda_{RED}}{\lambda_{NIR} + \lambda_{RED}}$$

where λ_{NIR} is the reflectance value of near-infrared (NIR), and λ_{RED} is the reflectance value of red.

To evaluate the homogeneity of the experimental sites, firstly, NDVI images were created, and then, cloud-covered and cloud-shadowed images were ignored by the Fmask algorithm [31]. Research and practical experience have shown that a stronger correlation existed between NDVI values and yield at certain phenological phases [32]. Consequently, only NDVI time points where the field-level NDVI values exceeded 0.7 were used for homogeneity analysis. Annual average NDVI values were calculated using 10×10 m Sentinel-2 raster data for the years from 2017 to 2021. Each year, the analysis included more than five images. The raster data were polygonized using ArcGIS Pro before analysis. To accurately assess NDVI within experimental plots, Sentinel-2 imagery (10 m resolution) was spatially intersected with plot polygons. Grids with less than 80% overlap were excluded to minimize mixed-pixel effects. Subsequently, average NDVI values were calculated for each plot and categorized into five classes (0.2 NDVI intervals) to highlight NDVI differences in the plot. While Experimental Site I exhibited a range of NDVI values, with 5.14% of pixels in the 0.6–0.8 category, all pixels at Experimental Site II were classified as high NDVI (0.8–1.0) (Figure 2).

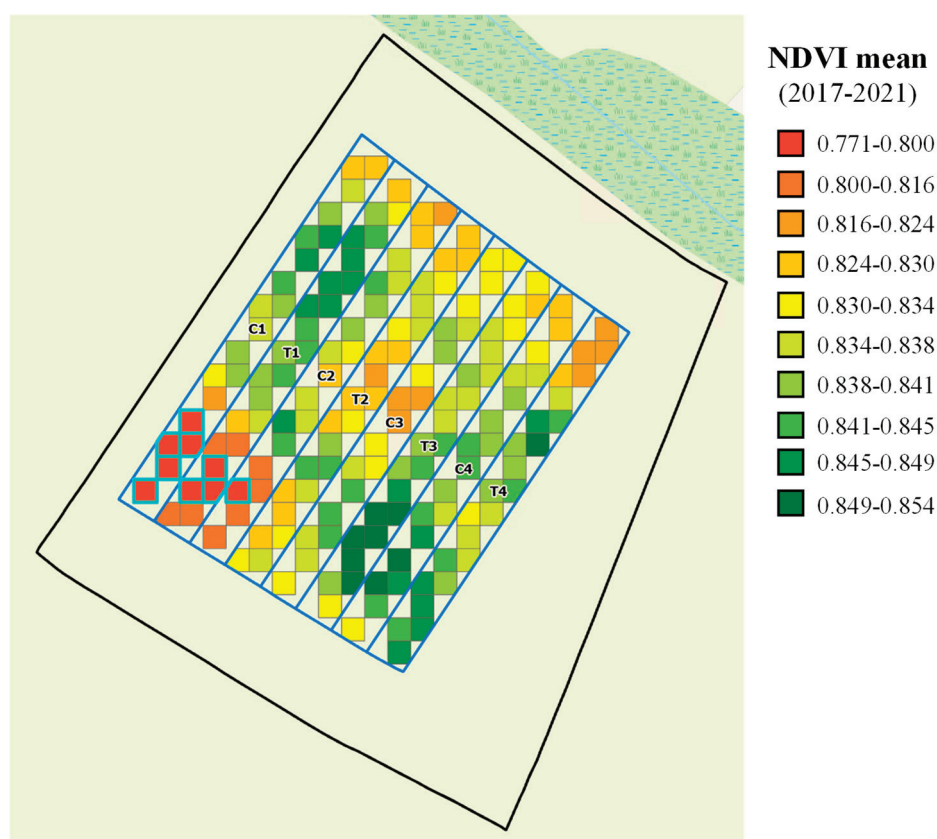


Figure 2. Average NDVI mean values with natural breaks coloring method, using 80% overlapping (Experimental Site I). Light blue polygon outline color shows the lower average NDVI values (below 0.8). C1–4 indicate control plots; T1–4 indicate Trichoderma-treated plots.

2.3. Measurements of Photosynthetic Potential and Plant Height

To assess photosynthetic potential, the number of viable leaves was counted, and chlorophyll content was measured weekly from treatment until harvest using a SPAD 502Plus chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan). At harvest, plant height for sunflowers was manually measured using a Bosch GR 500 (Robert Bosch Tools GmbH, Leinfelden-Echterdingen, Germany) leveling staff. Due to severe drought damage in 2022, maize plant height could not be assessed. For each plot, SPAD measurements

were taken on 20 randomly selected plants. Four points on four upper leaves of each plant were taken per plant and averaged. Viable leaf numbers were also counted on these same 20 plants. Plant height was measured similarly on 20 plants per plot right before harvest. Data from 80 control and 80 *Trichoderma*-treated plants were used to calculate the average and standard error for each data point.

2.4. Obtaining Harvest Data

The 2022 maize experiment harvest was gathered on 10 October 2022, while the 2022 and 2023 sunflower experiment harvests were gathered on 5 September 2022 and 13 September 2023, respectively. A John Deere S770i combine harvester equipped with an intelligent yield sensor, AutoTrac, and a StarFire 6000 RTK antenna (John Deere Inc., Mannheim, Germany) was used for all harvests. Yield and moisture content data were collected at one-second intervals and filtered using box plot analysis to eliminate outliers. Grid cells were excluded from analysis if they had less than 80% overlap with their assigned treatment area (Figure 2). Additionally, grid cells from Experimental Site I with predominantly low NDVI values (below a threshold of 0.8) were removed, as these areas were considered to have low productivity (Figure 2). Harvester data were spatially aligned with Sentinel-2 NDVI grids, and average yield and moisture values were calculated for each grid cell using the “Summarize Within” function in ArcGIS Pro.

2.5. Statistical Analysis

Data processing and visualization were performed using MS Excel 2016, while descriptive statistics and hypothesis testing were conducted in IBM SPSS 29. The normality assumption for parametric tests was assessed using Q–Q plots and the Shapiro–Wilk test. Homogeneity of variances (homoscedasticity) was evaluated using Levene’s test. When both assumptions were satisfied, the parametric *t*-test was employed. The non-parametric Mann–Whitney test was used for comparisons when the data did not meet the normality or homogeneity assumptions.

3. Results

The 2022 growing season was characterized by severe drought and elevated temperatures, particularly during the critical period from May to August (Figure 3).

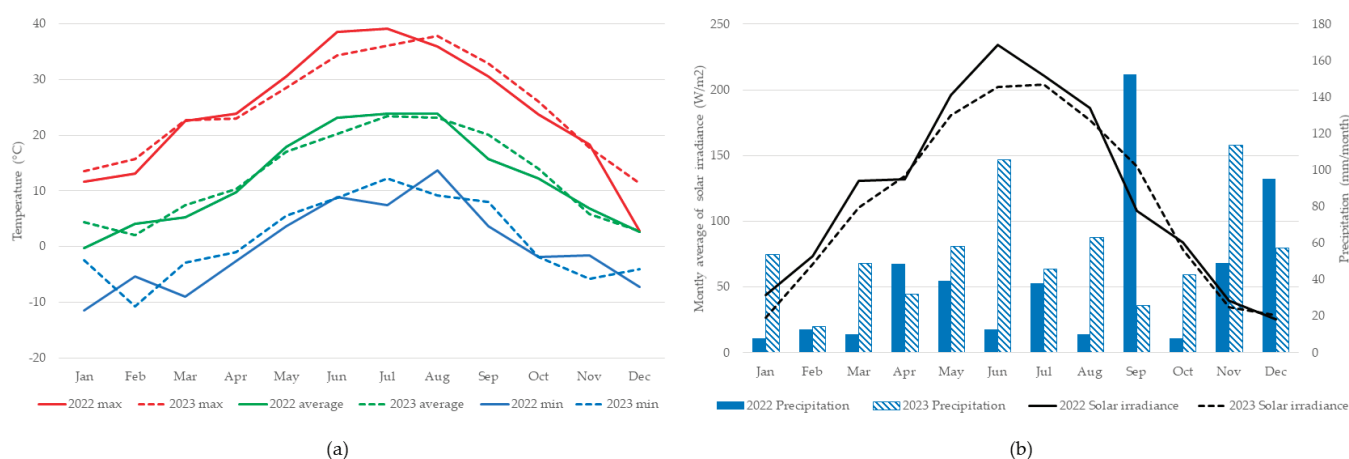


Figure 3. Monthly weather data of the experimental years 2022 and 2023. (a) Average, minimal and maximal temperatures. (b) Monthly average of solar radiation and precipitation.

The average annual precipitation is 600 mm in this region [33], but it was only 484.6 mm in 2022. A more abundant 662.2 mm precipitation was measured in 2023. Furthermore, the pre-sowing period (October 2021 to March 2022) was drier in 2022, with a precipitation deficit of 156 mm compared to the following year. The growing season of 2022

also had higher average temperatures (+1.5 °C) and increased solar irradiance compared to 2023 (Figure 3).

3.1. Homogeneity of the Experimental Sites

A five-year pre-experimental analysis revealed that all plots at Experimental Site II had highly stable and consistent NDVI values, ranging from 0.813 to 0.845 within the 0.8–0.9 category (Figure 2). However, at Experimental Site I, a small but significant proportion (5.5%) of plots showed lower NDVI values, falling within the 0.7–0.8 range. These plots were excluded from further analyses to ensure homogeneity within the dataset (Figure 2).

3.2. Plant Growth

Given the significant drought stress in 2022, which led to the premature death of corn plants, measuring plant height was not feasible. As the plants did not reach maturity, measuring height would not have provided meaningful data on growth vigor but rather on the timing of desiccation. The sunflower plants were shorter by 50 cm in 2022 compared to 2023. The application of *Trichoderma* species containing commercial products mitigated these drought effects, resulting in a 10 cm increase in sunflower height in 2022 and a 4 cm increase in 2023 (Table 1).

Table 1. Effects of *Trichoderma* TR04 and TR05 strains on the plant height of sunflower at harvest.

	Treatment	Sunflower (2022) (Mean ± SE)	Sunflower (2023) (Mean ± SE)
Plant height (cm)	<i>Trichoderma</i>	120.4 ± 2.14	174.9 ± 0.89
	Control	110.5 ± 3.37	171.1 ± 0.93
<i>t</i> -test <i>p</i>		0.014	0.003

3.3. Photosynthetic Potential

To evaluate the impact of treatment on photosynthetic potential, the number of viable (green) leaves was determined, and SPAD values were measured. Maize plants treated with *Trichoderma* spp. exhibited significantly increased leaf viability, with the effect becoming apparent as early as two weeks after the foliar application and persisting throughout the growing season (Figure 4a). The application of *Trichoderma* spp. to sunflower plants significantly increased viable leaf numbers, thereby stimulating plant development and prolonging leaf longevity. A statistically significant increase in leaf number was detected three weeks after treatment in 2023 and five weeks after treatment in 2022. This positive effect of *Trichoderma* spp. on leaf development and viability was maintained until the end of the vegetation period (Figure 4b).

Trichoderma treatment significantly increased SPAD values, indicating enhanced chlorophyll content in both maize and sunflower plants. Maize plants showed a rapid response to *Trichoderma* treatment, with increased SPAD values observed shortly after application. This positive effect persisted throughout the entire growing season (Figure 4c). Sunflower plants exhibited a delayed response, with significant increases in SPAD values observed five weeks post-treatment in 2022 and two weeks post-treatment in 2023 (Figure 4d).

While the foliar application of *Trichoderma* spp. had a positive impact on plant health and photosynthetic parameters, it did not significantly affect NDVI values. The difference in the NDVI between the treated and the control plots was less than 1%, following the treatment on either crop.

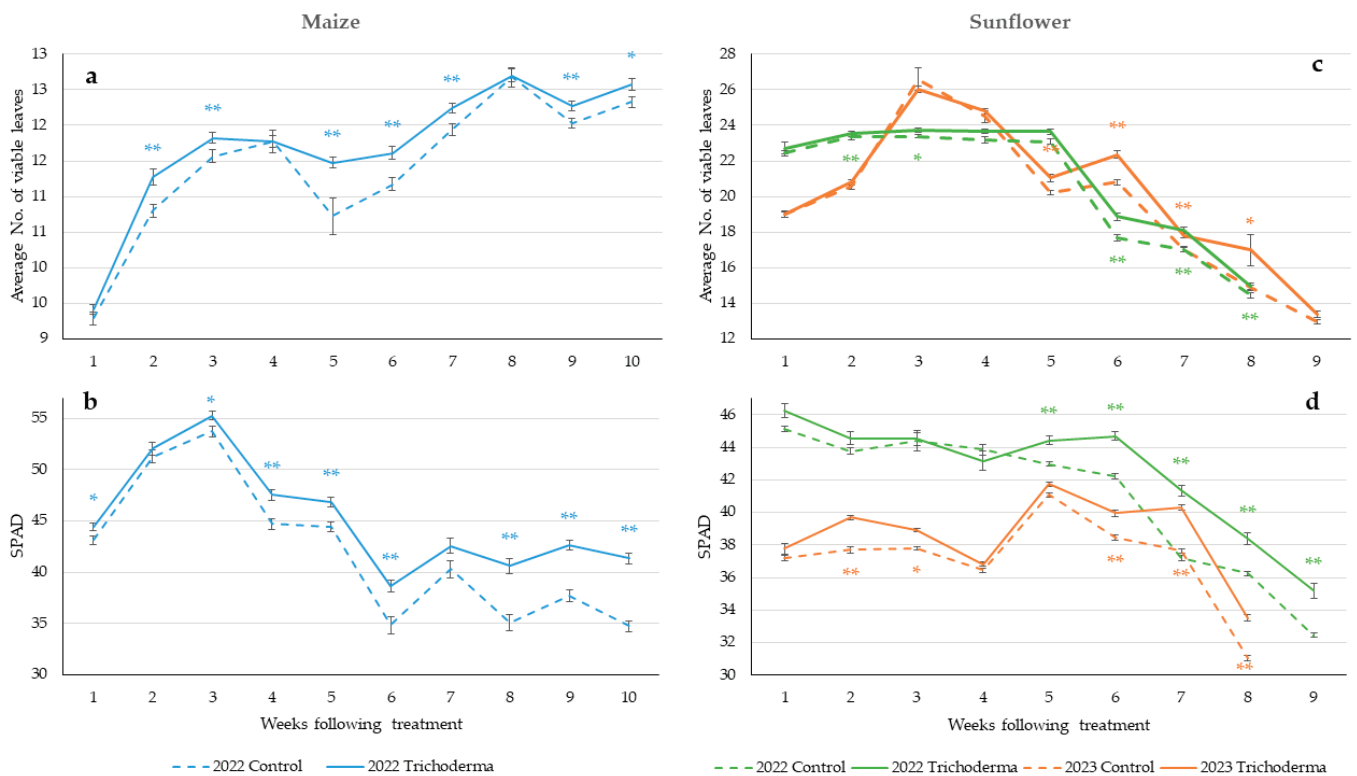


Figure 4. Changes in average number of viable leaves (a,c) and chlorophyll content (b,d, measured as SPAD values) of maize (a,b) and sunflower (c,d) plants following treatment with *Trichoderma* spp. compared to untreated control plants. Data points represent mean values, and error bars indicate standard error. *: t -test $p < 0.01$, **: t -test $p < 0.001$.

3.4. Yields and Moisture Content

The severe drought in 2022 significantly reduced maize yield to an extremely low 0.587 t/ha. However, *Trichoderma* treatment mitigated the negative effects of drought, significantly increasing maize yield to 1.62 t/ha (Figure 5a).

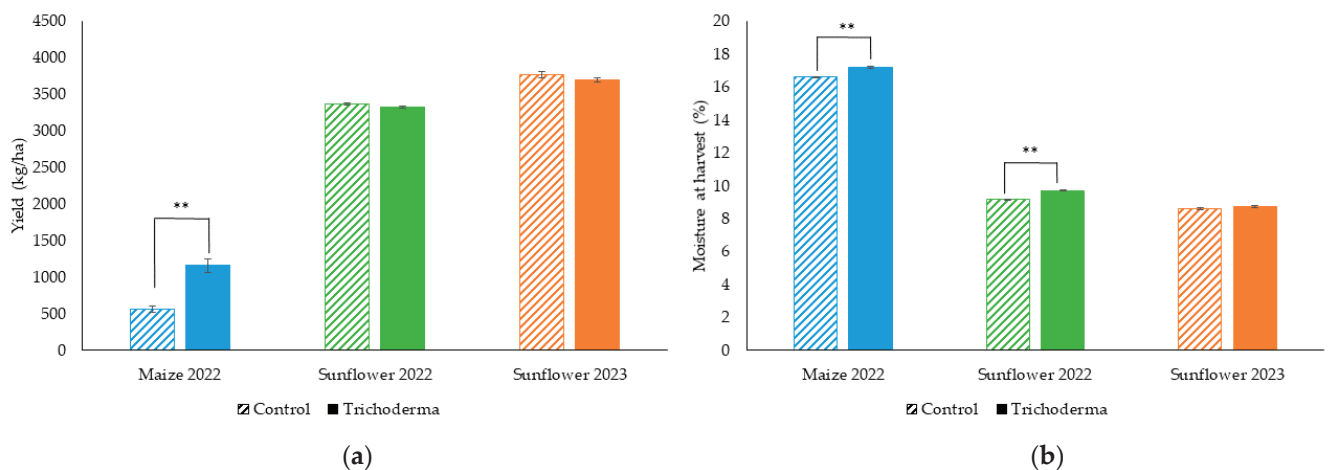


Figure 5. Average yield (a) and average moisture content of harvested seeds (b) of maize and sunflower plants treated with *Trichoderma* spp. compared to untreated controls. Error bars represent standard error. **: t -test $p < 0.001$.

The moisture content of the maize increased significantly from 16.6% to 17.19%, representing only a 0.59% difference between the treated and untreated maize. However,

this increase did not affect the quality of the harvested seeds. Similarly, in the drought year (2022), the moisture content of the sunflower increased slightly to 9.7% compared to the 9.16% measured in the seeds from the control plots, a difference of only 0.54%. In 2023, when normal precipitation was detected, neither the yield nor the moisture content of the sunflower showed any significant changes (Figure 5b).

4. Discussion

Climate change, with its increasing frequency and intensity of extreme weather events such as heatwaves and droughts, poses a critical threat to global food security by reducing crop yields and exacerbating pest and disease outbreaks [34,35]. To address these challenges, sustainable agricultural solutions, including the use of biostimulants, are emerging as viable alternatives to traditional chemicals [36,37]. While traditional agricultural practices often depend on chemical inputs, there is a growing demand for more sustainable and environmentally friendly approaches to secure food production in a changing climate [36,37].

Microbial biostimulants, particularly those based on *Trichoderma* species, have shown promise in mitigating the adverse effects of climate change on crop production [38]. These fungi form beneficial associations with plant tissues by producing plant hormones such as indole-3-acetic acid, gibberellic acid, abscisic acid, ethylene, jasmonic acid, and salicylic acid, as well as biostimulant metabolites like lactones and hydrophobins, which regulate growth and stress responses [18,39,40]. These interactions enhance root growth, improve water and nutrient absorption [41], and boost photosynthesis and carbohydrate metabolism, optimizing energy utilization for development [42–44]. *Trichoderma* spp. also produces siderophores, increasing iron availability crucial for metabolic functions, including photosynthesis [45]. Additionally, endophytic *Trichoderma* species have also been shown to induce systemic resistance, thereby enhancing their defense against pathogens and environmental stresses [27,40,46]. While the mechanisms of *Trichoderma* spp. in laboratory settings are well understood, its field application in crops like maize and sunflower remains underutilized. This is partly attributed to the difficulty in achieving field homogeneity, a critical factor for accurate evaluation of biostimulant efficacy in large-scale experiments evaluations [47]. Remote sensing technology employing vegetation indices, such as NDVI, can effectively assess field variability and inform experimental design, ultimately improving the reliability of field trials [48,49].

Maize and sunflower field crops were evaluated under homogeneous field conditions (5.7 and 11.3 ha, respectively). Field homogeneity was assessed using NDVI data from the previous five years. A preceding dry pre-sowing period (January–March 2022; 30.8 mm precipitation) compared to 2023 (117 mm), coupled with increased temperatures (+1.5 °C) and solar radiation during the 2022 growing season, contributed significantly to the severity of the drought. *Trichoderma* treatment significantly improved the plant's growth and photosynthetic capacity in sunflower plants. The increased number of viable leaves and higher SPAD index, indicative of enhanced chlorophyll content, were observed two weeks post-application and persisted throughout the growing season. These findings suggest that foliar *Trichoderma* treatment with endophytic strains can promote plant vigor and optimize photosynthetic efficiency. These differences highlight the sunflower's inherent drought tolerance due to its efficient water use and resilience mechanisms [50,51].

For field crops such as maize and sunflower, microbial biostimulants, including *Trichoderma* products, are typically applied through seed treatment or direct soil application of the root colonizing strains [25,52–55]. While these fungi are typically considered to colonize only the roots of plants [56], their presence has been detected in other plant tissues above the soil [57,58], suggesting that foliar spraying could also be an effective treatment method. While seed and soil treatments are common, foliar applications offer greater flexibility for targeted interventions during specific growth stages or in response to stress or control diseases. Disease symptom expression was not detected in the field of the studied crops. In vitro confrontation tests indicated high biocontrol indexes of both applied *Trichoderma*

strains toward several plant pathogen fungi [26]. Field tests necessary for the registration of the product with plant protection effect are underway. Preliminary results indicate the disease *Trichoderma* formulation tested in this study can repress the *Fusarium* head blight and sunflower foliar disease. The biocontrol activity of the product may further increase the yield and quality parameters of the harvested grains. Foliar spraying provides the potential for reducing environmental impact and operational costs by enabling combined applications with herbicides. However, ensuring compatibility between *Trichoderma* strains and herbicides is crucial for maintaining efficacy stressors [59].

In summary, microbial biostimulants, particularly *Trichoderma*-based products, represent a sustainable and flexible solution for enhancing crop resilience and productivity under drought stress, with further research needed to optimize their field applications and integration into agricultural practices.

5. Conclusions

Foliar spraying with the endophytic *Trichoderma* TR04 and TR06 strains stimulates abiotic stress tolerance of the monocot maize and the dicot sunflower under field conditions and would likely have the same effect with other annual crops as well. Pest control that includes spraying with low-cost microbial agents in combination with compatible pesticides (e.g., with post-emergent weed control) may lead to reduced costs and a smaller carbon footprint.

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Article

Growth Parameters, Yield and Grain Quality of Different Winter Wheat Cultivars Using Strip Tillage in Relation to the Intensity of Post-Harvest Soil Cultivation

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Abstract: The research has been undertaken to determine whether it is worthwhile to do a post-tillage on stubble before applying strip-till or whether tillage operations such as tillage and stubble ploughing should be performed. Therefore, ploughed tillage + strip tillage (PT), stubble disking + strip tillage (SD) and strip tillage (ST) operations were evaluated on three genetically distant winter wheat cultivars, including Formacja, Metronom and Desamo. A three-year field experiment was conducted from 2018 to 2021 at the Agricultural Experimental Station Kepa-Osiny in Pulawy, Poland. The experiment design was a split-block design with four repetitions of every treatment. The results showed that the cultivars differed in dry matter growth. However, no differences were found between the cultivar and post-harvest tillage method in terms of dry matter, plant height, and flag leaf area. Grain yield per ear was the main factor of yield variation across the cultivar and tillage systems. The extent of tillage only in the case of previously performed ploughing had an effect on the thousand grain weight. On the other hand, the omission of post-harvest tillage (ST) had a positive effect on the sedimentation index value. In terms of wheat grain yield, plough tillage (PT) proved to be the most advantageous, while reducing the intensity of tillage caused a systematic decrease in yield by 6% in the SD treatment and 9% in the ST treatment, respectively. Other quality parameters (gluten quantity, gluten index, falling number) did not depend on the applied tillage range. The response of cultivars to the applied cultivation methods was generally similar. Due to the beneficial effect of reducing the scope of cultivation on the environment, a small reduction in yield and no negative impact on the quality characteristics of grain, it is recommended to use strip-till cultivation without prior post-harvest cultivation. The results provide new insights into the growth of different winter wheat cultivars and the postharvest tillage applied, and they can be used in the future to validate existing wheat growth models.

Keywords: crop residue management; cultivation systems; dry plant mass; plant growth; yield structure; plant development

1. Introduction

Wheat is one of the most important cereal crops grown worldwide [1]. The global wheat sown area is around 214.3 million hectares. The European Union (EU) accounts for 22.9 million hectares, and Poland ranks third in terms of wheat production volume in the EU [2–4]. Due to the high popularity of the species, research is often conducted on the appropriate agrotechnology for this cereal. In recent years, research topics have focused on minimising the negative impact of agricultural production on the natural environment and on issues related to the potential of increasing carbon sequestration to limit the rate of climate change. This is the reason why research has concentrated in recent years towards reducing the use of plough tillage, whose role in carbon dioxide emission is very large [5].

Tillage also plays a decisive role in shaping plant growth conditions, which also directly determines the productive and economic effects obtained by the farmer. It can be

implemented in the traditional manner with full ploughing, but it can also be limited to shallow cover crops or eliminated altogether (direct drilling). Simplified tillage reduces negative environmental effects and increases the potential for carbon sequestration in the arable layer, but it also carries possible negative consequences in terms of lower yield [6–10]. Conservative cultivation systems, including strip tillage with stubble leaving, also influence the retention of nutrients in the soil and improve their balance, especially sulphur, which has a positive effect on the sulphur balance and the sustainability of agricultural production in soil management [11]. Ploughless cultivation, along with leaving as much crop residue as possible, can have a positive effect on soil quality, including the content of organic matter, and thus can increase the yield in wheat cultivation [12]. A particular cultivation method with the possibility of simultaneous sowing that combines the advantages of ploughing and no-till is strip-till [13]. There are reasonable views that the use of strip-till produces the same higher yields as plough tillage or zero tillage [14]. Advantages of this method include: aeration of the soil in the rhizosphere, faster heating of the soil in the strip-till, prevention of wind erosion, and a reduction in the loss of soil clay and silt particles—responsible for the soil sorption complex. Increased carbon and nitrogen content is also a beneficial effect of strip-till [15,16]. The wider row spacing used in this cultivation method may favour an increased grain yield as a result of a more efficient photosynthetic process in the plants [17].

The scientific literature on minimising plough tillage and replacing it with simplification or direct drilling is relatively rich, but most of the work published so far has dealt with the effects of different tillage methods on crop yields and quality. However, there is a lack of works that fully describe the process of shaping the final yield by assessing plant growth during the growing season in relation to the different cultivation methods. Only some works describe the growth of the root system and the aboveground weight of winter wheat in relation to tillage [18–20]. In addition, Fu et al. [21] noted that monitoring plant growth and development in a given environment is an important way to understand phenomena occurring in the soil environment. In the case of wheat, cultivation intensity and cultivar choice have been found to have some influence [22,23]. The rate of biomass growth has a direct impact on wheat grain yield and the carbon cycle [24–28]. Thus, it can be said that the determination of temporal and spatial variability of aboveground plant weight in wheat provides basic information on plant growth, but furthermore allows estimation of potential yields in a given growing season [29–31]. Conventional methods for assessing plant growth involving sampling green plants per unit area and drying them and later assessing the dry matter yield are extremely time- and labour-intensive [26,27,32], and hence, there are not many examples of work using such methods in the world literature. Therefore, this study aimed to determine the growth parameters of winter wheat grown using the strip-till method in relation to the extent of post-harvest tillage and cultivar. The research hypothesis was that both the extent of post-harvest tillage applied and the cultivar would significantly affect the plant growth rate, which would ultimately determine the level of grain yield obtained.

2. Materials and Methods

2.1. Field Conditions and Setup of the Experiment

The research hypothesis was verified by field experiments carried out in the three growing seasons: 2018/2019, 2019/2020, 2020/2021. The experiment was located at the Agricultural Experimental Station Kępa-Osiny (51°27' N; 22°2' E) belonging to the Institute of Soil Science and Plant Cultivation-State Research Institute in Pulawy-Poland. The soil was classified as a Gleyic Phaeozems (according to the World Reference Base for Soil Resources). Winter wheat was used as a forecrop in each year.

The field experiment was set up in the split-block method in four replications for each treatment. Three different tillage methods were applied according to Figure 1. The depth of plough cultivation was 20 cm, of the cultivator 8–10 cm, and in strip-till cultivation in the cultivation strips 18–20 cm. The distance between two rows of plants in the cultivated strip was 12 cm and the distance between the planting strips was 36 cm. The second

experimental factor featured three wheat cultivars of considerably varying origin (from different breeders). They were selected taking into account all available varietal traits, mainly the resistance to biotic and abiotic stresses. The characteristics of the selected cultivars are presented in Figure 2.

CHARACTERISTICS OF THE POST-HARVEST CULTIVATION IN THE VARIANTS USED IN THE EXPERIMENT

1.- Ploughed tillage + strip tillage (PT)

plow post-harvest medium-deep ploughing, followed by strip tillage combined with wheat sowing



Image from an experimental plot Ploughed tillage + strip tillage (PT). Crop residues have been ploughed in and are no longer on the soil surface.

2. - Stubble discing + strip tillage (SD)

post-harvest stubble loosening with a disc harrow, followed by strip tillage combined with wheat sowing



Image from an experimental plot Stubble discing + strip tillage (SD). The stubble has been disturbed, but crop residues are still on the soil surface.

3. Strip tillage (ST)

without any post-harvest cultivation, only strip-till cultivation was carried out together with wheat sowing



Image from an experimental plot Strip tillage (ST). The stubble remains were not disturbed in the post-harvest cultivation, there are much more of them than in the SD treatment

Figure 1. Characteristics of treatments using different post-harvest cultivation.

Characteristics of winter wheat varieties used in the study

FORMACJA	METRONOM	DESAMO
BREEDING COMPANY: Poznańska Hodowla Roślin	BREEDING COMPANY: RAGT	BREEDING COMPANY:DANKO
❖ quality variety, group A	❖ quality variety, group A	❖ quality variety, group A
❖ very high yield potential	❖ good and very stable yield,	❖ high yield potential and very high profitability of cultivation;
❖ good resistance to fungal diseases	❖ does well in all soils and climate conditions,	❖ useful for intensive cultivation technology on medium-quality and good soils at different sowing dates;
❖ good tolerance to soil acidification	❖ high tolerance to soil acidification allows cultivation in all locations,	❖ good disease resistance;
❖ medium plants with good resistance to lodging	❖ good plant health,	❖ earing date – medium early;
❖ good frost resistance	❖ good winter hardiness.	❖ winter hardiness: – very good.
❖ exceptional tillering strength		
❖ 1000 grain mass and high uniformity		
good resistance to sprouting in the spike		

Figure 2. Characteristics of the cultivars used in the research.

In each year of the research, the same setup was used with a combination of different types of cultivation and cultivar. There were four replicates of every treatment. Each plot measured 9 m width and 25 m length, which corresponded to 225 m². The detailed setup is shown in Figure 3.

The content of nutrients and pH of soil are presented in Figure 4. The detailed agri-technical calendar is shown in Figure 5.

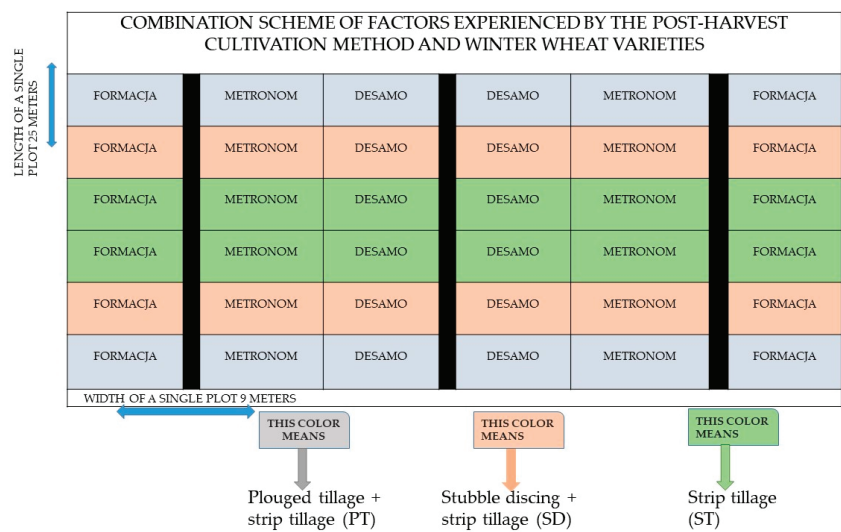


Figure 3. Plan of the applied experiment and combination of factors.

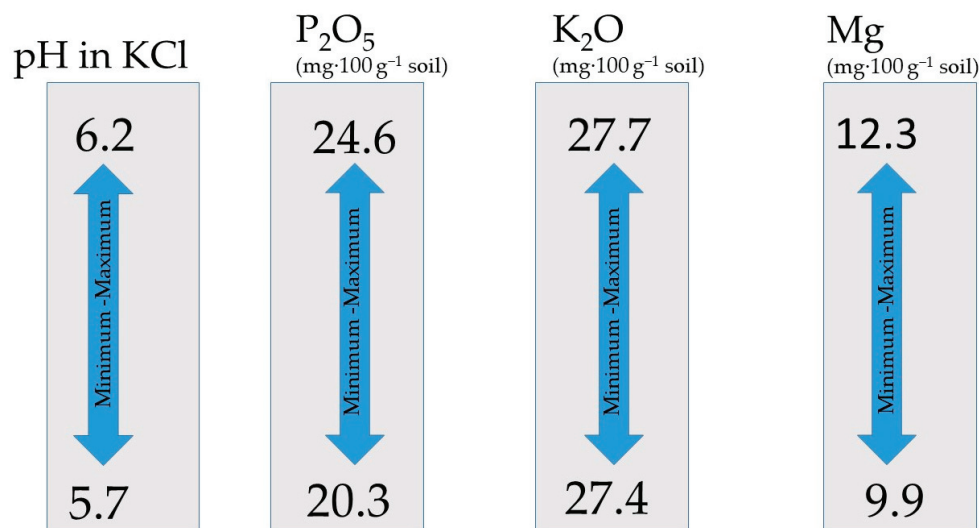


Figure 4. Characteristics of the physicochemical properties of soil.

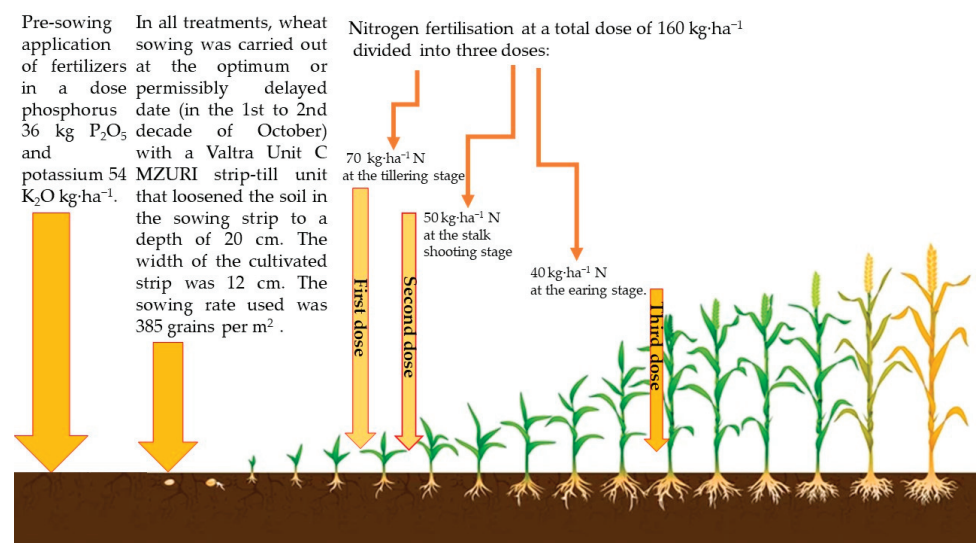


Figure 5. Agricultural technology and fertilisation used in the conducted research.

2.2. Dry Matter Yields at Selected Growth Stages

To assess adhesion parameters, plants were sampled from an area of 1 m², from each plot in duplicate, at the following stages

- Bush stage BBCH 29
- Stalking BBCH 32
- Flowering BBCH 59

Prior to placing the samples in the dryer, 10 plants were randomly selected from each sampled plot for measurement of height and flag leaf area, using an Area Meter AM 101 automatic leaf scanner from BioScientific LTD, Hoddesdon, UK. All green matter sampled from each plot was placed in an air-circulating dryer for 72 h, at 55 °C, and then weighed.

Meteorological conditions during the vegetation period of the plants were characterised by mean daily temperature (°C) and precipitation (mm), as well as the perennial averages of these parameters (Table 1).

Table 1. Meteorological conditions during the growing seasons in which the study was conducted.

Month	Temperature °C				Precipitation (mm)			
	Growing Season			Multi-Year Average	Growing Season			Multi-Year Average
	2018/2019	2019/2020	2020/2021		2018/2019	2019/2020	2020/2021	
September	15.5	14.4	14.9	13.3	48.0	57.8	102.0	55.0
October	10.0	10.8	10.4	8.0	40.5	33.5	90.0	44.0
December	4.2	6.4	5.1	2.7	8.9	31.4	14.0	39.0
November	0.9	3.1	1.7	−1.4	61.0	47.9	19.0	37.0
January	−2.4	1.7	−1.4	−3.3	62.0	27.1	51.0	31.0
February	2.9	3.4	−2.7	−2.3	15.2	56.5	38.0	30.0
March	5.7	4.7	2.8	1.6	20.9	16.7	12.0	30.0
April	10.0	8.9	6.9	8.7	39.0	14.4	50.0	39.0
May	13.9	11.9	12.9	14.5	69.0	93.9	61.0	58.0
June	22.7	19.1	20.0	17.2	37.0	159.0	53.0	65.0
July	19.4	19.3	22.2	19.5	71.0	31.9	110.0	80.0
August	20.4	20.3	17.1	17.8	94.3	95.5	219.0	87.0

During the study, weather conditions were varied between years. Autumn and winter periods saw large differences in temperature, while meteorological conditions in the spring months were similar, not differing largely from the multi-year average. Each season saw periods with greater or lesser precipitation deficiency, but in general, precipitation totals in each season were relatively high. This was particularly the case in the third season 2020/2021, when precipitation in the preharvest was well above the multi-year average.

2.3. Yield Structure

At full maturity, plants were taken from an area of 1 m², two samples from each plot; thus, each treatment was represented by 8 samples in order to determine the yield components. The number of plants and the number of ears were determined in the samples. The number of grains per ear and grain weight per ear were determined in each sample on 10 randomly selected plants. The harvest index was calculated by the division of the grain yield by the sum of the grain yield and straw weight and was expressed in percentage.

Harvest was performed with a combine in the first decade of August, at the stage of full maturity. Following the harvest, grain moisture was determined at 15% moisture content.

2.4. Grain Quality

In order to determine the quality of the grain, representative samples of grain were taken after harvesting, in which the following were determined: the thousand grain weight and the bulk density of the grain (using a densimeter equipped with a 1000 mL

cylinder—according to the PN-EN ISO 7971-3 standard [32]), and the quality characteristics of the flour were determined, such as: the wet gluten amount (according to standard PN-A-74042) [33], the gluten index (GI), the falling number and the Zeleny sedimentation index (according to standard PN-EN ISO 5529) [34].

2.5. Statistical Analysis of the Results

The obtained results were developed using analysis of variance (ANOVA) and the Tukey test at a significance level of $p \leq 0.05$ with STATISTICA ver. 13.1 software (StatSoft, Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Effects of Tillage Types on Wheat Growth Parameters

Plant growth and final yield are the product of a number of processes, the most important of which is the intensity of photosynthesis, which determines the rate of growth of vegetative mass, which is essential for an adequate supply of essential assimilates to the plant during the generative phase [35]. The accumulated higher vegetative green matter of wheat increases the efficiency of sunlight utilisation, which contributes to a better use of the photosynthetic process in yield [36]. The lack of adequate green matter at any given time increases the danger of severe stress conditioning, e.g., shoot reduction and reduced ear number in wheat [37]. A fast rate of green matter growth is also important due to the higher coverage and increased competitiveness of wheat against weeds, which is also conditioned by the varietal factor [38]. It should be added that the rate of green matter accumulation is also dependent on the ability to take up nutrients from the soil with water. In the present study, the rate of green matter accumulation varied according to the cultivation method. The lowest value of this trait at the tillering stage was found in the PT plot with ploughing, while the highest was in the ST plot without post-tillage. The differences between the above-mentioned treatments exceeded 6% and were, therefore, quite large, although statistically insignificant. This indicates that the use of the plough had a negative effect on plant growth, but only in the initial period of plant development because in subsequent stages, significantly higher weight per unit area was found in the treatments with ploughing. The SD and ST treatments did not differ significantly throughout the growing season with respect to the trait in question (Table 2), so it can be concluded that the conditions for plant growth in these treatments were similar. Research by Lipiec and Nosalewicz [39] showed that the building of aboveground green matter in winter wheat depends on soil compactness and water availability. These researchers concluded that higher soil compaction has a positive effect on dry matter building in winter wheat. The results of our own research did not confirm this because it was on ploughed soil that by far the largest amount of green matter was obtained. Sha et al. [40] showed that colder and more compacted inter-row soil in strip-till cultivation was unfavourable for early root growth, resulting in limited shoot and green matter growth in maize. The fact that wheat seed sowing with a strip-till unit in ST under pre-applied ploughing conditions was slightly deeper than in the treatments with the other cultivation treatments may also have had an influence on the growth conditions associated with better water availability. Such a relationship was shown by Ali et al. [41], who found that wheat grown in furrows, where the rows into which plants were sown, gathered water and accumulated green matter better than plants grown without furrows, on a flat surface. Although, in the study described, this positive effect appeared with a delay—only at stages associated with intensive weight gain.

Table 2. Green dry matter of wheat plants (g·m²) at different developmental stages according to cropping system, cultivar, and years of study.

Specification	Development Phase		
	Tillering	Stem Formation	Beginning of Earing
Cultivation system			
Ploughed tillage + strip tillage (PT)	97 ^a	434 ^a	1207 ^a
Stubble discing + strip tillage (SD)	101 ^a	413 ^{a b}	1151 ^b
Strip tillage (ST)	103 ^a	405 ^b	1157 ^b
Cultivar			
Formacja	90 ^b	413 ^b	1181 ^a
Metronom	116 ^a	431 ^a	1222 ^a
Desamo	105 ^b	408 ^b	1112 ^b
Years			
2019	85 ^b	358 ^c	1329 ^a
2020	88 ^b	501 ^a	987 ^c
2021	128 ^a	394 ^b	1196 ^b
Factor interaction			
T	ns	*	*
C	***	ns	***
Y	***	***	***
T × C	ns	ns	**
T × Y	***	*	**
C × Y	**	***	**
T × C × Y	**	*	***

Different letters (^{a–c}) mean the significant difference ($p \leq 0.05$) according to ANOVA and Tukey's test. Significant interaction on level p value * ≤ 0.05 , ** ≤ 0.01 , *** < 0.001 , and ns—not significant difference, T—tillage, C—cultivar, Y—year.

As expected, the effect of the cultivar on plant growth rate, as measured by the amount of dry matter per unit area, was also significant. At each growth stage, the highest value for this trait was recorded in the Metronom cultivar. The cultivars Desamo and Formacja had significantly less green matter. However, there was no interaction between the cultivar and the cultivation method, indicating that despite the large variation between cultivars, their response to the cultivation method was the same. Saini et al. [42] identified cultivars that were more efficient in tolerating reduced to zero tillage among the wheat and rice cultivars tested. The reasons for the variation in the response of cultivars to cultivation conditions may be very complex. It appears that the pool of free carbohydrates may play an important role in this regard, allowing plants with a larger supply of carbohydrates to survive the stress better. The size of the root system may also play an important role here. This was pointed out by Tazhibayeva et al. [43], who showed that the development of the root system varies between cultivars and may determine more efficient water uptake under drought conditions and thus mass accumulation. This was also confirmed by results obtained by other authors [17,44–46].

Kumar and Sachan [2], as well as Wilczewski et al. [47], pointed out the large role of mulch in no-tillage in shaping soil moisture. In the studies of these authors, no-tillage and direct seeding into the mulched soil surface had a more beneficial effect on wheat yield than ploughing and irrigation. In the present study, mulch on the soil surface existed in large amounts only in the inter-rows. The strongest surface coverage of mulch (cut straw) was in the inter-rows in the ST, where the degree of soil cover was about 50%. At the SD treatment,

the amount of mulch remaining on the surface was already considerably less (around 15%). It seems that the positive effect of mulch on wheat growth and yield was limited due to the relatively high rainfall during the years of the study, which meant that the reduction in water loss from the mulched surface did not have a clearly positive effect in shaping plant growth parameters. Sha et al. [48] found that strip-till plants had the ability to rapidly adapt and recover from abiotic stresses due to which green matter was comparable in strip-till and plough tillage. Our research showed that the system of applied post-harvest cultivation together with strip tillage had a significant interaction in combination with the accumulated green mass, but only in two phases—the stem formation and the beginning of earing. In the case of cultivars, a highly significant interaction (p value < 0.001) was found in the tillering and the beginning of earing phases. Years also had a significant interaction in each phase studied (p value < 0.001). Interactions between individual experimental factors were also significant. We found a non-significant interaction only between $T \times C$ in two developmental phases: tillering and stem formation. Rieger et al. [49] also showed that the cultivation system had no significant interaction with the green mass of plants, which we also noted in our own studies. Plaza-Bonilla et al. [50] also demonstrated the lack of interactions, indicating that green matter and root matter develop the same in different tillage systems.

The flag leaf is an organ that plays a very important role in shaping the assimilation process and, consequently, the yield of wheat plants. Its larger surface area promotes a higher intensity of photosynthesis-related processes [51,52]. In our study, neither the crop used, nor the cultivar had a significant effect on this trait of wheat plants (Table 3). However, a strong effect of years related to the occurrence of different weather conditions was found. As indicated by Yang et al. [53], the area of the flag leaf depends precisely on weather conditions and is smaller under stress conditions. In particular, drought stress associated with water scarcity negatively affects flag leaf area in wheat [54,55]. The lack of differences in the trait in question according to the experimental factors suggests that the magnitude of drought stress was similar in all treatments. We found a significant interaction in flag leaf area and other factors only between years (Y), and also between factors $C \times Y$ and $T \times C \times Y$.

Table 3. Flag leaf area (cm²) according to cultivation method, cultivar and years of study.

Specification	Flag Leaf Area (cm ²)
Cultivation system	
Ploughed tillage + strip tillage (PT)	22.5 ^a
Stubble discing + strip tillage (SD)	22.7 ^a
Strip tillage (ST)	23.2 ^a
Cultivar	
Formacja	25.5 ^a
Metronom	25.3 ^a
Desamo	24.9 ^a
Years	
2019	20.1 ^c
2020	23.2 ^a
2021	21.6 ^b

Table 3. *Cont.*

Specification	Flag Leaf Area (cm ²)
Factor interaction	
T	ns
C	ns
Y	***
T × C	ns
T × Y	ns
C × Y	*
T × C × Y	*

Different letters (^{a–c}) mean the significant difference ($p \leq 0.05$) according to ANOVA and Tukey's test. Significant interaction on level p value * ≤ 0.05 , *** < 0.001 , and ns—not significant difference, T—tillage, C—cultivar, Y—year.

The height of the plants at the different phases depended significantly on the cultivation method used. Wheat plants were highest at the stem formation and earing stages in the ST treatment (Table 4). Plant height, on the other hand, did not depend significantly on the cultivar, although it is worth noting the slightly higher height of the Metronom cultivar at the tillering and stem formation stages. This trend was reversed at the beginning of earing when plants of the Metronom cultivar were characterised by the lowest height compared to the other cultivars. It is a well-known fact that plants vary greatly depending on the weather conditions specific to the vegetation period. Künze et al. [56] also showed a significant effect of years on plant height than the cultivar itself. In our own research, the colder April in 2021 could have slowed down plant growth and accelerated the course of further phenological phases associated with the photoperiod, which could have resulted in a lower plant height this year compared to 2019 and 2020. The research by Wilhelm et al. [57] confirms such a relationship, which was found in our own research. All of the experimental factors, T, C and Y, showed highly significant interactions (p value < 0.001) with plant height in individual phases. We also found highly significant interactions between factors C × Y; C × Y and T × C × Y (p value < 0.001). We did not find a significant interaction only between T × C in the stem formation phase.

Table 4. Winter wheat plant height (cm) at different developmental stages according to cropping system, cultivar and years of study.

Specification	Development Phase		
	Tillering	Stem Formation	Beginning of Earing
Cultivation system			
Ploughed tillage + strip tillage (PT)	27.3 ^a	51.2 ^a	86.9 ^b
Stubble discing + strip tillage (SD)	26.9 ^a	50.6 ^a	86.3 ^b
Strip tillage (ST)	27.7 ^a	53.4 ^a	90.1 ^a
Cultivar			
Formacja	26.2 ^a	50.9 ^a	89.3 ^a
Metronom	28.6 ^a	52.2 ^a	85.4 ^a
Desamo	26.6 ^a	50.5 ^a	93.2 ^a

Table 4. Cont.

Specification	Development Phase		
	Tillering	Stem Formation	Beginning of Earing
	Years		
2019	27.0 ^a	50.6 ^b	91.2 ^b
2020	25.9 ^a	64.7 ^a	97.1 ^a
2021	19.0 ^b	41.1 ^c	61.2 ^c
	Factor interaction		
T	***	***	***
C	***	***	***
Y	***	***	***
T × C	*	ns	***
T × Y	***	***	***
C × Y	***	***	***
T × C × Y	***	***	***

Different letters (^{a–c}) mean the significant difference ($p \leq 0.05$) according to ANOVA and Tukey's test. Significant interaction on level p value * ≤ 0.05 , *** < 0.001 , and ns—not significant difference, T—tillage, C—cultivar, Y—year.

3.2. Effects of Tillage Types on Wheat Yield

Grain weight was significantly higher for the PT treatment compared to the SD and ST treatments (Table 5). The other two treatments showed no significant differences between them. Straw weight was also significantly higher in the PT treatment than in the SD and ST treatments, by 9.5 and 10.1%, respectively.

The varietal factor significantly differentiated wheat grain yields. Formacja showed a significantly higher grain yield than cultivar Desamo. In contrast, the cultivars showed no significant differences between each other in terms of straw weight. The harvest index value is a trait that determines the ratio between accumulated vegetative green matter and the main yield, which in the case of wheat is grain [58]. White and Wilson [59] indicate that the limiting harvest index (HI) value for wheat to guarantee the highest possible yield is 0.55. In their study, this value was slightly lower and did not depend on the cropping system and cultivar. In some studies [60], the role of cultivation system and cultivar in shaping the HI was greater than in our study. Grain mass $\text{g} \cdot \text{m}^{-2}$ showed a highly significant interaction between T and Y (p value < 0.001), and a significant interaction between C (p value ≤ 0.05). Straw mass had a highly significant interaction with Y (p value < 0.001) and a significant interaction with T (p value ≤ 0.01). In addition, there was a highly significant interaction between the factors T × Y (p value < 0.001), and a significant interaction between C × Y (p value ≤ 0.05). In the Harvest index, we also found a highly significant interaction between C and Y (p value < 0.001). We also found a high interaction between T × Y and C × Y (p value < 0.001).

The role of the tillage method in shaping cereal yields may result from its influence on plant density [61]. In the presented study, however, the range of tillage applied did not significantly affect the size of this wheat canopy trait (Table 6), although it is undoubtedly noteworthy that there was a tendency for the number of plants per unit area to decrease in the treatment with the least intensive tillage (ST). Similarly, Wesołowski and Cierpiąła [62] showed a lower wheat grain yield when post-harvest tillage was reduced in winter wheat cultivation. Also, the genetic factor did not have a significant effect on this wheat canopy trait, although the tendency towards a lower value of this trait in the Formacja cultivar was quite pronounced. However, the main role in shaping grain yield, according to cultivar, was

played by yield per ear, which varied a lot over the years—a generally known relationship resulting from the influence of weather conditions on this trait [63–65].

Table 5. Grain weight, straw and harvest index value according to crop, cultivar and years of study.

	Grain Weight g·m ^{−2}	Straw Weight g·m ^{−2}	Harvest Index (%)
Cultivation system			
Ploughed tillage + strip tillage (PT)	970.7 ^a	953.2 ^a	0.51 ^a
Stubble discing + strip tillage (SD)	886.3 ^b	891.9 ^{a b}	0.50 ^a
Strip tillage (ST)	881.0 ^b	861.8 ^b	0.51 ^a
Cultivar			
Formacja	955.0 ^a	887.4 ^a	0.52 ^a
Metronom	916.4 ^{a b}	890.7 ^a	0.51 ^a
Desamo	866.7 ^b	928.8 ^a	0.49 ^a
Years			
2019	1027.0 ^a	981.4 ^b	0.52 ^a
2020	925.6 ^b	1066.4 ^a	0.47 ^b
2021	785.5 ^c	659.0 ^c	0.55 ^a
Factor interaction			
T	***	**	ns
C	*	ns	***
Y	***	***	***
T × C	ns	ns	ns
T × Y	ns	***	***
C × Y	ns	*	***
T × C × Y	ns	ns	ns

Different letters (a–c) mean the significant difference ($p \leq 0.05$) according to ANOVA and Tukey's test. Significant interaction on level p value * ≤ 0.05 , ** ≤ 0.01 , *** < 0.001 , and ns—not significant difference, T—tillage, C—cultivar, Y—year.

In a study by Parylak and Pytlarz [66], limiting wheat pre-sowing cultivation to the use of a cultivating unit resulted in high yield decreases, compared to plough tillage, but it should be noted that the mentioned researchers used sowing by the traditional method, i.e., with a standard seed drill. Jaskulska et al. [61] achieved similar results to our study in terms of winter wheat yield depending on the tillage method. These authors also used a Mzuri unit for setting up the experiment and compared this treatment to conventional ploughing and simplified (no-plough) tillage, in which sowing was conducted with a seed drill. Similar results were also obtained by Mohammadi et al. [67]. It should be emphasised that in the strip till method, the soil in the sowing strip is cultivated to a depth of 20 cm, i.e., to a depth appropriate for ploughing and even deeper, which lowers the negative effects associated with too much soil compaction that occurs in direct sowing (sowing without prior tillage). Therefore, on the basis of the present research, it may be concluded that the strip-till method in the conditions of the clay soil, in which our research was conducted, does not create the same conditions for plant growth as with properly conducted ploughing, but the negative effects associated with its non-application are the same.

Table 6. Yield and its structure according to the crop used, cultivar and years of study.

Specification	Tillering Index	Plant Density (pcs·m ⁻²)	Number of Ears (pcs·m ⁻²)	Weight of Kernels per Ear (g)	Yield (t·ha ⁻¹)
Cultivation system					
Ploughed tillage + strip tillage (PT)	2.2 ^a	284 ^a	477 ^a	1.87 ^a	7.88 ^a
Stubble discing + strip tillage (SD)	1.9 ^a	279 ^a	478 ^a	1.63 ^b	7.41 ^b
Strip tillage (ST)	2.1 ^a	267 ^a	490 ^a	1.62 ^b	7.16 ^b
Cultivar					
Formacja	2.1 ^a	273 ^a	469 ^a	1.75 ^a	7.68 ^a
Metronom	2.1 ^a	278 ^a	488 ^a	1.78 ^a	7.53 ^a
Desamo	2.2 ^a	282 ^a	488 ^a	1.59 ^b	7.24 ^b
Years					
2019	1.6 ^c	310 ^a	505 ^a	1.82 ^a	8.10 ^a
2020	2.3 ^b	260 ^b	482 ^b	1.64 ^b	7.73 ^b
2021	2.5 ^a	262 ^b	458 ^b	1.66 ^b	6.62 ^c
Factor interaction					
T	ns	ns	ns	***	ns
C	ns	ns	ns	***	ns
Y	***	***	***	***	ns
T × C	ns	ns	ns	**	ns
T × Y	*	ns	*	ns	ns
C × Y	*	ns	***	ns	ns
T × C × Y	*	ns	ns	*	ns

Abbreviation ‘pcs’ means ‘pieces’. Different letters (^{a–c}) mean the significant difference ($p \leq 0.05$) according to ANOVA and Tukey’s test. Significant interaction on level p value * ≤ 0.05 , ** ≤ 0.01 , *** < 0.001 , and ns—not significant difference, T—tillage, C—cultivar, Y—year.

Among the studied yield structure traits, only for the weight of kernels per ear (g) was a highly significant interaction (p value < 0.001) term was found between T and C. We found a highly significant interaction between Y (p value < 0.001) and all yield structure traits except the grain yield itself. C × Y; C × Y; T × C × Y showed significant interaction ($p \leq 0.05$) with each other in terms of tiling index. The number of ears was influenced by the interaction between T × Y ($p \leq 0.05$) and C × Y (< 0.001). The weight of kernels per ear (g) showed a significant influence between experience factors T × C (p value ≤ 0.01) and T × C × Y (p value ≤ 0.05). Roohi et al. [68] indicate that the interaction between the cultivation system and the cultivar in the yield concerns the grain head from the spike, which was confirmed in our own research. Herrera et al. [69] compared the available scientific studies and showed that the percentage of reported significant T × C interactions was higher for spring wheat (71%) than for winter wheat (40%).

3.3. Effects of Tillage Types on Wheat Grain Quality

In our own research, it was found that the cultivation method as well as the cultivar had a significant effect on the thousand grain weight (TGW). The highest value of this grain trait was found under post-harvest plough tillage (PT) and the lowest was when strip tillage was combined with sowing made in no-till (ST) (Table 7). The higher grain weight and yield in the plough system relative to the no-till system were also found by other authors [70,71], but it should be noted that they did not use a strip-till unit for sowing. The

effect of the cultivar on thousand grain weight was also significant. The highest thousand grain weight was found in the Metronom cultivar and the lowest in the Desamo cultivar. An important indicator of grain quality, which determines its maturity, is its bulk density. This trait, which determines its milling value, did not significantly depend on the post-harvest tillage applied, although it should be emphasised that the tendency for a higher value of this trait in the PT treatment was clear. Among the winter wheat cultivars tested, we found a significant effect on the density of grain at the bulk state, as each cultivar differed significantly in this respect. The Formacja cultivar had the highest value for this trait, which was 4% higher than that of Metronom and 9% higher than that of Desamo. Some scientific studies have shown that the value of winter wheat grain density is higher when plough tillage is applied than under reduced tillage conditions [72]. In contrast, Jaskulska et al. [8] and Taner et al. [73] showed no effect of using the tillage system (ploughing, reduced tillage and no-till) on this grain trait. Large differences between cultivars in grain density were pointed out by many authors [74–76]. Bobryk-Mamczarz et al. [77] pointed out the influence of weather conditions varying over the years on this trait, which was also confirmed by their own research, as the grain density values obtained in each year of the study differed significantly. The tests carried out showed that the method of post-harvest cultivation had no statistically significant effect on the amount of total gluten. Only a statistically insignificant tendency towards a slightly higher amount of gluten in the grain from the ST treatment was found. The results obtained in our study are in line with what was obtained in their studies by Šíp et al. [78] and Woźniak and Rachoń [79]. In studies by other authors, the effect of applied soil tillage intensity in shaping the amount of gluten varied. Amato et al. [80] showed a higher amount of gluten in grain from treatments with more intensive (plough) cultivation, while Konavko and Ruža [81] showed the opposite relation, i.e., a higher amount of gluten in wheat grain from treatments with less intensive cultivation. Our own research showed that the amount of gluten was dependent on the winter wheat cultivar. The cultivar Desamo had the statistically significant highest amount of gluten. On average, the Metronom cultivar contained 5% less gluten and the Formacja cultivar 14% less. There is a consensus among various authors dealing with the issue of wheat grain quality that the amount of gluten is a varietal trait [82–84]. A very extensive study in this field on 762 cultivars, also confirming the mentioned relationship, was conducted by Pengpeng et al. [85]. No less important than the cultivar in shaping the amount of gluten is the variable weather conditions over the years. This is confirmed by our own research. Irrespective of cultivation method and cultivar, on average, the highest amount of gluten was found in 2020, and in the other two years, 2019 and 2021, 6.3% and 3.0% less, respectively (Table 7). The important role of years in shaping the amount of gluten was also emphasised by other authors [86,87]. This is related to the weather-dependent efficiency of nitrogen use for protein synthesis in grain [88–90]. The intensity of the post-harvest tillage applied did not significantly affect the gluten index value (Table 7). In contrast, a study by Gawęda and Haliniarz [70] showed that tillage intensity can significantly modify this trait. The authors cited found higher gluten index values under no-tillage compared to plough tillage. A different relationship—a higher gluten index under reduced tillage than under no-tillage—was found by Buczek et al. [91]. In our study, a strong influence of the varietal factor on the gluten index was shown. The Formacja cultivar had by far the highest value (81% on average) of the trait in question. In the other two cultivars, the gluten index was significantly lower. The gluten index is cultivar-related in winter wheat [92], which was also confirmed in our own research. Our own research also showed, the effect of years on the gluten index value. The lowest value of this trait was found in 2020, while the other years did not differ significantly in the value of this trait (Table 7). In general, there is some consensus in the literature on the large effect of weather condition variance over the years, with this being explained by drought stress, the occurrence of which reduces the value of this trait [93]. The sedimentation index relates to both the quality and quantity of protein in the grain, and therefore has an impact on the quality of the bread obtained and, in particular, its structure [94]. The highest possible value is

desirable. A high sedimentation rate should be combined with a high content of gluten proteins, especially gluten, which is the most important for baking [95]. Our study showed a significant effect of the extent of post-harvest cultivation on the Zeleny sedimentation coefficient. Its highest value was found in the ST treatment, in which strip-tillage with seed sowing was carried out directly into the stubble. Significantly lower values for this parameter were found in the reduced tillage (SD) and plough tillage (PT) treatments, by 8 and 13%, respectively. Similar trends in the effect of tillage intensity on sedimentation rate were also found by Bilalis et al. [96] and Wozniak and Rachoń [79]. In the study of Šíp et al. [78], on the other hand, the sedimentation rate was higher with a plough tillage system than with no-tillage.

Table 7. The value of the analysed grain quality traits depending on the post-harvest cultivation used, cultivar and year of research.

Specification	Thousand Grain Weight (g)	Bulk Density of Grain (kg·hl ^{−1})	Amount of Gluten (%)	Gluten Index (%)	Sedimentation Index Zeleny (cm ³)	Falling Number (s)
Cultivation system						
Ploughed tillage + strip tillage (PT)	39.2 ^a	74.3 ^a	33.7 ^a	66 ^a	46 ^c	358 ^a
Stubble discing + strip tillage (SD)	37.9 ^b	72.1 ^a	33.6 ^a	66 ^a	49 ^b	361 ^a
Strip tillage (ST)	36.1 ^c	71.9 ^a	34.0 ^a	63 ^a	53 ^a	374 ^a
Cultivar						
Formacja	37.9 ^b	76.0 ^a	30.9 ^c	81 ^a	47 ^b	365 ^b
Metronom	41.5 ^a	72.7 ^b	34.2 ^b	59 ^b	59 ^a	321 ^c
Desamo	33.8 ^c	69.5 ^c	35.9 ^a	55 ^c	42 ^c	404 ^a
Years						
2019	37.9 ^a	72.3 ^b	30.5 ^c	68 ^a	39 ^c	371 ^a
2020	37.8 ^a	69.5 ^c	36.8 ^a	62 ^b	60 ^a	363 ^a
2021	38.0 ^a	77.6 ^a	33.7 ^b	66 ^a	49 ^b	358 ^a
Factor interaction						
T	***	**	ns	*	***	ns
C	***	***	***	***	***	***
Y	ns	***	***	*	***	ns
T × C	**	ns	ns	***	ns	ns
T × Y	ns	ns	ns	ns	**	ns
C × Y	***	***	***	ns	***	***
T × C × Y	ns	ns	ns	*	*	ns

Different letters (^{a–c}) mean the significant difference ($p \leq 0.05$) according to ANOVA and Tukey's test. Significant interaction on level p value * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , and ns—not significant difference, T—tillage, C—cultivar, Y—year.

In our study, there was a significant effect of cultivar on the Zeleny sedimentation coefficient. The highest value was found in the Metronom cultivar, which had a higher index than the Formacja cultivar by 20% than on the Desamo cultivar by 29%. The strong influence of the cultivar on the value of the sedimentation index was indicated by many authors [79,95,97–99]. In addition, our study found a large variation in sedimentation rate values between years (Table 7), the highest being recorded in 2020, with significantly lower values in the other years. Tatar et al. [100] consider that the magnitude of the sedimentation index is conditioned by the occurrence of drought at the grain pouring stage. In the cited study, the sedimentation rate was significantly lower under rainfall deficit conditions. The falling number is considered to be an important discriminator

for the technological value of the grain, which determines alpha-amylase activity. The minimum value of the falling number according to wheat standards (EN ISO 3093) is 250 [101]. In our study, the tested grain from each research treatment met this requirement. However, there was no statistically significant effect of the extent of post-harvest tillage on the falling number value. Weber [86] and Buczek et al. [91] found that plough tillage favoured a higher fall number compared to reduced tillage. On average, the highest falling number was characterised by the cultivar Desamo (independent of the treatment, more than 400), but in the case of the other two cultivars, the values of this trait were also high and exceeded 300. Similarly, a large role of the cultivar in shaping the falling number was shown by Knapowski et al. [102] and Amiri et al. [83]. In our own study, there was no significant effect of the study years on the value of falling number. However, the literature indicates that weather conditions have the greatest influence on the value of this trait. The lack of heavy rainfall in the pre-harvest period favours a high value of the fall number [103]. The tillage system showed significant interaction (p value < 0.001) between the thousand grain weight and Zeleny's sedimentation index, the bulk density of grain (p value < 0.001) and gluten index ($p \leq 0.05$). The cultivar showed a high significant interaction (p value < 0.001) with each of the tested grain quality traits. Years of research had a high significant interaction (p value < 0.001) with the bulk density of grain, amount of gluten and Zeleny's sedimentation index, and also significant interactions with the gluten index ($p \leq 0.05$). A highly significant interaction (p value < 0.001) was also confirmed between the $C \times Y$ experience factors on traits such as thousand grain weight, bulk density of grain, amount of gluten, Zeleny's sedimentation index and falling number. $T \times C$ had a significant interaction for gluten index (p value < 0.001) and thousand grain weight (p value ≤ 0.01). $T \times Y$ had a significant interaction for Zeleny's sedimentation index (p value ≤ 0.01). Interactions between $T \times C \times Y$ had a significant impact (p value ≤ 0.05) on gluten index and Zeleny's sedimentation index. Buczek et al. [91] study of the interaction between T, C and Y showed a highly significant interaction of Zeleny's sedimentation index and falling number and gluten index only for T and C, which was also confirmed by our own study.

4. Conclusions

The lowest value of this trait at the tillering stage was found in the ploughed tillage + strip tillage treatment. In the subsequent growth phases, this was the treatment with the highest aboveground weight, indicating that the plant growth rate was clearly higher in this treatment. The genetic factor had a significant influence on plant growth rate. In each growth phase, the highest value for this trait was recorded in the Metronom cultivar. The cultivars Desamo and Formacja had a significantly lower green matter. The results of the present study can be used to validate existing wheat growth models. The extent of harvest tillage preceding the strip-till sowing of wheat had a significant effect on the thousand grain weight. The higher value of this trait was characterised by grain obtained from the treatment in which strip-tillage of wheat was applied after ploughing. The grain quality parameters (gluten content, gluten index, falling number) did not depend on the applied post-harvest tillage regime, except for the sedimentation index. The beneficial effect of strip-till cultivation on the environment and the slight decrease in yield and no effect on quality characteristics mean that we recommend the use of strip-till cultivation without prior post-harvest cultivation, but it is important to select the appropriate winter wheat cultivar.

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Article

Impacts of Weed Resistance to Glyphosate on Herbicide Commercialization in Brazil

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Abstract: Herbicides are essential tools for the phytosanitary security of agricultural areas, but their excessive use can cause problems in agricultural production systems and have negative impacts on human health and the environment. The objective of this study was to present and discuss the main causes behind the increase in herbicide commercialization in Brazil between 2010 and 2020. Data from the Brazilian pesticide database, provided by the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA)*, were used. In 2010 and 2020, Brazil sold 157,512 and 329,697 tons of herbicide active ingredients, respectively, representing a 128.1% increase in commercialization over 11 years. Some herbicides, such as clethodim, haloxyfop-methyl, triclopyr, glufosinate, 2,4-D, diclosulam, and flumioxazin, showed increases in sales volumes between 2010 and 2020 of 2672.8%, 896.9%, 953.5%, 290.2%, 233.8%, 561.3%, and 531.6%, respectively, percentages far exceeding the expansion of Brazil's agricultural area. The primary reason for this sharp increase in herbicide sales was the worsening cases of weeds resistant and tolerant to glyphosate, with species such as *Conyza* spp., *Amaranthus* spp., *Digitaria insularis*, and *Eleusine indica* standing out. This situation created the necessity of the use of additional herbicides to achieve effective chemical control of these weed species.

Keywords: chemical control; genetically modified crops; integrated management; pesticide market

1. Introduction

Herbicides are a class of pesticides used to control weeds, serving as a crucial tool in integrated management [1,2], particularly in large-scale agricultural operations. Their importance lies in the fact that, without weed control, agricultural activities suffer severe damage, leading to significant yield losses that can even make crop harvests unfeasible [3,4]. On a global scale, inefficient weed control causes yield losses of approximately 32% across various crops [5]. In this context, herbicide application aims to protect crops from interference by weed communities [6,7], which tend to occur at higher densities in agricultural areas and exhibit superior adaptation to the competitive process for essential natural resources [8]. Weeds can also serve as alternative hosts for insect pests and plant disease-causing microorganisms [9,10] and exhibit allelopathic activity [11,12].

Due to their efficacy and ease of application, allowing high operational efficiency [13], herbicide use has rapidly intensified in Brazil's agricultural, forestry, and pasture areas. The widespread adoption of chemical control also results from the labor shortage in rural areas caused by the ongoing migration to urban centers, driving a preference for herbicide use over manual weed control [14]. In recent decades, glyphosate has become prominent

among herbicides registered in Brazil, primarily due to its favorable agronomic characteristics, such as broad-spectrum activity [15,16], effectiveness in controlling weeds at advanced developmental stages [17], efficiency in managing perennial species that propagate vegetatively [18,19], and the absence of soil active residues [20,21]. Two transformative events in Brazilian agriculture have driven the significant increase in glyphosate use. The first was the introduction and rapid adoption of the no-tillage system in grain production areas, where pre-sowing burndown became a routine operation, initially with glyphosate as the main herbicide used for this purpose [22]. The second event was the introduction of genetically modified (GMO) cultivars, such as soybean, corn, and cotton, which were engineered to be glyphosate-resistant [23].

The lack of or insufficient rotation of herbicides with different modes of action, along with the failure to implement integrated weed management principles, led to excessive selection pressure for glyphosate on the weed community [24,25]. Over years of intensive glyphosate use, weed biotypes that are resistant to this herbicide were selected, spreading to the point that glyphosate-resistant populations are now found throughout Brazil [26,27]. Currently, there have been official reports of 20 cases of weeds with simple or multiple resistance to glyphosate [28]. Resistant biotypes identified in Brazil include the following species: *Lolium perenne*, *Conyza bonariensis*, *Conyza canadensis*, *Digitaria insularis*, *Conyza sumatrensis*, *Chloris elata*, *Amaranthus palmeri*, *Eleusine indica*, *Amaranthus hybridus*, *Euphorbia heterophylla*, *Echinochloa crus-galli*, and *Bidens subalternans* [29–32]. In addition to these species, a noteworthy challenge is the management of volunteer glyphosate-resistant corn plants, where grain losses during corn harvests have led to infestations in subsequent soybean crops, complicating chemical control [33,34].

The emergence and spread of resistant biotypes across various regions of Brazil necessitated the use of additional herbicides to complement glyphosate's action on previously controlled species [35]. Among the herbicides that began to be more intensively used as a result of the spread of resistant biotypes in Brazil's grain production areas are 2,4-D, triclopyr, diclosulam, flumioxazin, haloxyfop-methyl, clethodim, and glufosinate. Despite the increase in resistant populations, glyphosate remains the primary active ingredient used in Brazilian agriculture, with variations in formulations depending on the type of salt utilized.

Thus, the objective of this study was to present and discuss data on herbicide commercialization in Brazil, with the main hypothesis being that the increase in herbicide consumption is driven by the worsening cases of glyphosate-resistant weeds.

2. Materials and Methods

The primary data used for the analysis of pesticide commercialization and the number of formulated products per active ingredient were obtained from the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA), the agency responsible for releasing commercialization data in Brazil. According to Brazilian legislation, manufacturing and importing companies must report their annual sales volume to IBAMA, which compiles this information to produce and publish reports on pesticide sales in the country. The data collection period from IBAMA covered from 2010 to 2020. After analyzing the primary database, it was necessary to make adjustments and standardize the historical series to align it with the guidelines of the Food and Agriculture Organization of the United Nations (FAO) [36] and the Organisation for Economic Co-operation and Development (OECD) [37].

To normalize and standardize the data from the IBAMA primary database, the following data were excluded: (1) The volume of non-agricultural pesticide (NA) sales in Brazil between 2010 and 2020. (2) The sales volume of products registered exclusively as adjuvants or surfactants (2010 to 2017), which were no longer classified as pesticides in Brazil following the *Ministério da Agricultura e Pecuária* (MAPA) act nº 104 of 20 November 2017, published in the Official Gazette of 21 November 2017. From 2018 onward, these products were no longer included in IBAMA's database, and by excluding

data from 2010 to 2017, the historical series was standardized. (3) The sales volume of products classified as semiochemicals and microbiologicals (2010 to 2013). Since 2014, IBAMA began reporting these biopesticides separately from chemical, biochemical, and phytochemical products in its bulletins, and (4) sales of formulated pesticide products between industries in Brazil (2010 to 2020), because counting inter-company sales could result in double counting.

Additionally, after direct consultation with FAO and OECD technicians and statisticians, who also develop and disseminate international databases related to agricultural sustainability indicators, the following standardizations were implemented: (1) For the herbicides 2,4-D, glyphosate, picloram, MCPA, aminopyralid, florpyrauxifen-benzyl, fluroxypyr, haloxyfop-methyl, imazapic, imazaquin, imazapyr, imazethapyr, triclopyr, and cyhalofop-butyl, the data were recorded in acid equivalent (a.e.). These herbicides are formulated with different derivatives to ensure formulation stability, where standardizing the data in a.e. allows normalization regardless of the derivative used in the formulation. (2) For the herbicides diquat and paraquat, the data were recorded in tons of ion equivalent. Similar to the herbicides mentioned in Item 1, these compounds are also formulated with derivatives, and data standardization ensures consistent reporting. (3) For the fungicides copper hydroxide, copper oxychloride, copper oxide, and copper sulfate, the data were recorded in tons of metallic copper equivalent to maintain consistency, and (4) for all other pesticides, the data were recorded in tons of active ingredient (a.i.).

The increase in herbicide sales in Brazil from 2010 to 2020 was compared with the growth of the Brazilian agricultural area (perennial and temporary crops) and soybean area, using data from the SIDRA system, provided by the *Instituto Brasileiro de Geografia e Estatística* (IBGE) [38]. Additionally, it was compared with the expansion of non-degraded pasture areas (pastures that effectively use agricultural inputs, such as herbicides), calculated using data from the Image Processing and Geoprocessing Laboratory of the *Universidade Federal de Goiás* (LAPIG/UFG) [39].

3. Results and Discussion

3.1. Pesticide Sales in Brazil (2010 to 2020)

The data presented in Table 1 show the evolution of pesticide sales in Brazil, divided by usage classes, from 2010 to 2020. During this period, herbicides accounted for the highest sales volume among pesticides used in Brazilian agriculture. In 2010, 157,512 tons of herbicide active ingredients were sold in Brazil, representing 53.34% of all pesticides (chemical, biochemical, and phytochemical) commercialized that year. However, by 2020, herbicide sales had reached 329,697 tons of active ingredients, marking a 128.11% increase over 11 years and accounting for 58.89% of total pesticide sales in Brazil. For comparison on a global scale, in 2019 herbicides represented approximately 52% of the total pesticide market share [40], which is slightly lower than the Brazilian percentage. Additionally, the global increase in herbicide consumption from 2010 to 2020 was 29.92% [36], significantly lower than the increase recorded in Brazil.

Two reasons can be given to justify the use of higher quantities of herbicides compared to other pesticide categories. The first reason is that fungicides and insecticides are typically applied during the crop cycle (from sowing to harvest), whereas herbicides are used before, during, and after the crop cycle [41]. Their use is common before crop establishment (pre-sowing burndown) and after harvest (burndown of spontaneous vegetation during the fallow period). The second reason is the limited availability of bioherbicides in the Brazilian market, unlike bioinsecticides and biofungicides, which are already well-established in Brazilian agriculture. This has led to the exclusive use of chemical molecules for weed control in agricultural areas. Bioherbicides can be formulated with microorganisms or phytotoxins derived from microorganisms, insects, or plant extracts that act as natural agents for weed control [42].

Table 1. Sales data and representative percentage of each pesticide class (2010 to 2020). Grouping standard proposed by OECD [37].

Year	Total	Insecticides ¹	Fungicides ²	Herbicides	Others ³
Tons of Active Ingredient ⁴					
2010	295,287	72,856	63,365	157,512	1554
2011	314,862	77,950	61,298	173,171	2443
2012	383,999	75,146	55,117	251,094	2642
2013	407,792	96,135	53,541	256,004	2112
2014	420,467	104,172	61,028	252,765	2502
2015	445,684	95,636	74,354	272,852	2842
2016	451,276	86,627	85,605	276,147	2897
2017	453,111	86,174	90,882	273,502	2553
2018	490,495	83,711	110,698	293,053	3033
2019	564,635	98,562	132,357	329,697	4019
2020	610,098	101,783	144,241	359,308	4766
Percentage (%) of total sales in the year					
2010	100.00	24.67	21.46	53.34	0.53
2011	100.00	24.75	19.47	55.00	0.78
2012	100.00	19.57	14.35	65.39	0.69
2013	100.00	23.57	13.13	62.78	0.52
2014	100.00	24.78	14.50	60.12	0.60
2015	100.00	21.46	16.68	61.22	0.64
2016	100.00	19.20	18.97	61.19	0.64
2017	100.00	19.02	20.06	60.36	0.56
2018	100.00	17.07	22.57	59.74	0.62
2019	100.00	17.46	23.44	58.39	0.71
2020	100.00	16.68	23.64	58.89	0.79

¹ Includes insecticides, acaricides, molluscicides, and nematocides. ² Includes fungicides, bactericides, and pesticides used in seed treatment. ³ Includes fumigants, growth regulators, and seed protectants. ⁴ For the herbicides 2,4-D, glyphosate, picloram, MCPA, aminopyralid, florypyrauxifen-benzyl, fluroxypyr, haloxyfop-methyl, imazapic, imazaquin, imazapyr, imazethapyr, triclopyr, and cyhalofop-butyl, data are reported in tons of acid equivalent. For the herbicides diquat and paraquat, data are reported in tons of ion equivalent. For fungicides such as copper hydroxide, copper oxychloride, copper oxide, and copper sulfate, data are reported in tons of metallic copper equivalent. All other pesticides are reported in tons of active ingredients.

Regarding pesticide sales data, the introduction of *Bt* technology in soybean, corn, and cotton cultivars, combined with the increased use of bioinsecticides (both microbiological and macrobiological), likely contributed to the reduced share of chemical insecticides in the Brazilian pesticide market [43,44]. On the other hand, the growing share of fungicides in Brazil can be attributed mainly to the following factors: (1) weakening of some breeding programs focused on disease resistance/tolerance, resulting in the release of cultivars susceptible to various diseases and requiring more chemical control intervention; and (2) increased pathogen resistance to fungicides, leading to a significant rise in the use of multi-site fungicides in preventive applications, particularly in crops like soybeans [45,46]. It is worth noting that the recommended dose per area for multi-site fungicides is significantly higher than for more recently introduced site-specific fungicides.

Several factors explain the increased use of herbicides during the studied period (2010 to 2020) in Brazil: (1) expansion of no-till farming areas [47,48], where herbicide applications for pre-sowing burndown are common, with higher doses often used due to the more advanced growth stages of weeds. Brazilian farmers have become more aware of soil conservation [49], leading to the replacement of mechanical weed control practices, such as plowing, harrowing, and scarifying, which expose the soil to erosion; (2) growth in second-crop corn areas [50], where farmers use herbicides for pre-harvest soybean desiccation to advance corn sowing, in addition to increased herbicide use in corn crops themselves; (3) reduction in rural labor availability, which hinders the use of manual weed control methods [51]. Data from the World Bank Group [52] show that

the rural population in Brazil decreased by 31.58% between the years 2000 and 2020; (4) lack of bioherbicides or microbiological herbicides on the Brazilian market, unlike the growing availability of bioinsecticides and biofungicides; and (5) greater availability of products formulated from the same herbicide active ingredient, increasing competition among manufacturers/importers, which may have driven prices down for some herbicides. Examples of this price reduction can be found in the database of the *Companhia Nacional de Abastecimento* (CONAB) [53]: The herbicide clethodim (same formulated product and same active ingredient concentration) was sold for USD 51.41 per liter in 2010 and was marketed for USD 8.45 per liter in 2020 (based on data from Paraná State, Brazil). The herbicide haloxyfop (same formulated product and same active ingredient concentration) was sold for USD 32.82 per liter in 2010 and was marketed for USD 9.92 per liter in 2020 (based on data from São Paulo State, Brazil). (6) Increased areas infested with herbicide-resistant weed biotypes, particularly for glyphosate [54–56].

Herbicide-resistant weeds are the most significant factor behind the increased herbicide sales and are the central theme of the present material. It is important to note that the rise in herbicide sales does not necessarily indicate abusive behavior by farmers. Unlike other pesticides, there is often resistance among agricultural experts to increasing herbicide doses, as this could increase the risk of crop injuries, prolong residual herbicide activity, and cause carryover problems for subsequent crops. Additionally, higher herbicide doses raise production costs.

In addition to evaluating the commercialization of herbicides, it is relevant to investigate whether the environmental hazard potential profile of these products changed during the analyzed period. In Brazil, IBAMA is the institution responsible for assessing pesticides based on the environmental risks associated with their use, categorizing them into four classes: Class I: Highly hazardous to the environment; Class II: Very hazardous to the environment; Class III: Hazardous to the environment; and Class IV: Slightly hazardous to the environment. To determine these classifications, IBAMA evaluates various factors, such as bioaccumulation, toxicity to non-target organisms, biodegradation, and soil mobility. In 2010, 0.001% of the herbicides sold in Brazil were classified as Class I, while 18.90% belonged to Class II and 81.10% to Class III. These proportions changed minimally by 2020, with 0.02% of the herbicides classified as Class I, 20.63% as Class II, and 79.35% as Class III. Notably, during this time frame, no herbicides classified as Class IV were sold in Brazil.

Regarding the human toxicity potential of herbicides sold in Brazil, it is important to highlight that between 2010 and 2020, no herbicides classified by the World Health Organization [57] as Ia (extremely hazardous) or Ib (highly hazardous) were marketed. An integrative analysis of the consolidated database showed that not all herbicide active ingredients experienced increased sales in Brazil from 2010 to 2020. Therefore, this study presents and discusses the sales results for herbicides that saw the highest sales growth during this period and are recommended for use in grain production areas.

3.2. Evolution of Herbicide Sales in Brazil (from 2010 to 2020)

The evolution of 2,4-D herbicide sales (tons of acid equivalent) is represented in Figure 1A, where a nearly linear increase from 2010 to 2020 can be observed. The herbicide 2,4-D is a synthetic auxin, characterized by causing various metabolic disturbances in sensitive plants, such as abnormal growth, death of root tissues, epinasty, and obstruction of phloem [58]. In Brazil, the herbicide 2,4-D is primarily marketed in the form of amine salt and is indicated for the control of broadleaf weeds [59]. In Brazil, 15,323 tons of this herbicide's active ingredient were sold in 2010, while in 2020, this number rose to 51,149 tons, representing an increase of 233.8%. The observed increase in usage exceeds the increase recorded during this 11-year period concerning the area of agriculture designated for harvesting (annual and perennial crops) (24.3%), the soybean area (59.4%), and the area of pastures without degradation (16.0%), which can be classified as the most demanding agricultural activities for 2,4-D in the country (Figure 1B).

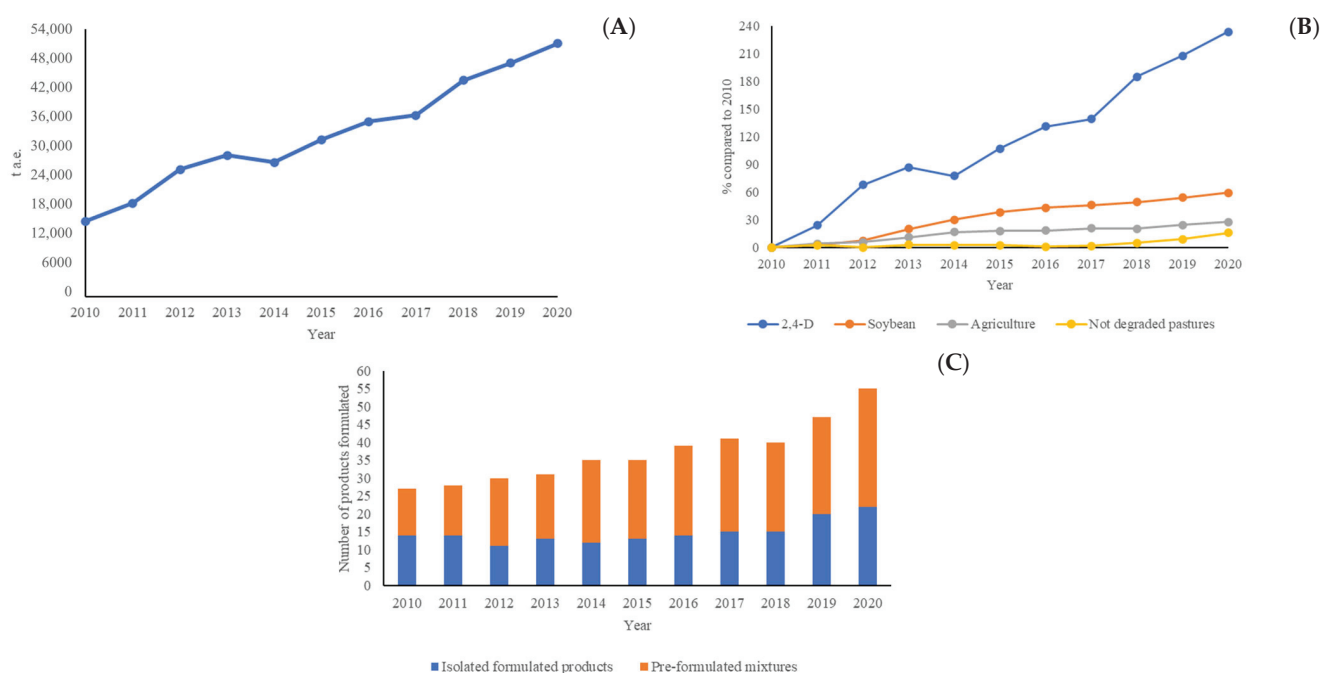


Figure 1. Sales of 2,4-D (tons of a.e.) from 2010 to 2020 (A). Evolution of herbicide sales in relation to the year 2010, compared to the evolution of soybean area (IBGE), the area of agriculture designated for harvesting (annual and perennial) (IBGE), and the area cultivated with pastures without degradation (LAPIG/UFG) (B). Number of formulated products based on 2,4-D sold in Brazil (C) (period from 2010 to 2020).

The main reason for the increased use of 2,4-D during this period is the worsening cases of glyphosate-resistant weed biotypes, specifically the increasing infestation of biotypes from different *Conyza* spp. species and areas infested with resistant *Amaranthus* spp. biotypes [27,60]. As a result, glyphosate, which previously provided effective control of these weed species, began requiring complementary herbicides during pre-sowing burndown to help manage resistant biotypes, driving the sales of 2,4-D in Brazil. It is important to note that the Enlist™ technology, which enables the selective use of 2,4-D, glyphosate, and glufosinate in crops like soybean and corn [61], was not available in the Brazilian market during the evaluated period (2010 to 2020) and, therefore, cannot be considered one of the factors responsible for the observed increase in use.

Other factors may have also contributed to this rise in 2,4-D consumption in Brazil, including: (1) increased use of herbicides in pastures between 2010 and 2020 [62]; (2) expansion of no-till farming in Brazil, as the main agricultural use of this herbicide is linked to pre-sowing burndown, especially before soybean crops; (3) increased availability of 2,4-D products in the Brazilian market. In 2010, 27 formulated products containing the active ingredient 2,4-D were sold, 14 containing only 2,4-D (as a single active ingredient) and 13 pre-formulated mixtures with other active ingredients. By 2020, the number of formulated products containing 2,4-D had increased to 55 (22 single active ingredient products and 33 pre-formulated mixtures), representing a 103.7% increase in product availability (Figure 1C); (4) expansion of agricultural areas infested with glyphosate-tolerant weeds, such as *Commelina benghalensis*, *Spermacoce latifolia*, *Ipomoea* spp., and *Tridax procumbens* [63,64]; and (5) shorter plantback intervals for soybean sowing compared to other auxin herbicides.

Another auxin herbicide, triclopyr, also experienced an increase in sales between 2010 and 2020 (Figure 2A). In 2010, 228 tons of triclopyr (acid equivalent) were sold, while in 2020, this volume increased to 2405 tons, representing a 953.5% increase in sales. This rise far exceeds the increase in soybean area (59.4%) and the area of pastures without degradation (16.0%), two agricultural activities where triclopyr is potentially used (Figure 2B). The main causes for the increase in triclopyr sales from 2010 to 2020 are as follows: (1) worsen-

ing cases of glyphosate-resistant weed biotypes, particularly the increased infestation of *Conyza* spp. biotypes, including those resistant to 2,4-D (rapid necrosis) [65], where triclopyr became a tool for management [66], and (2) expansion of areas infested with species from the genera *Spermacoce*, *Borreria*, and *Mitracarpus*, with triclopyr becoming one of the go-to herbicides for controlling these species during the off-season. Other less impactful factors may have also contributed to the rise in triclopyr sales, such as (1) increased availability of triclopyr-based products in the Brazilian market, especially products formulated with triclopyr as the sole active ingredient (a 400% increase in the availability of single-active ingredient formulations from 2010 to 2020) (Figure 2C), and (2) lower antagonism when mixed with ACCase-inhibiting herbicides compared to 2,4-D [67].

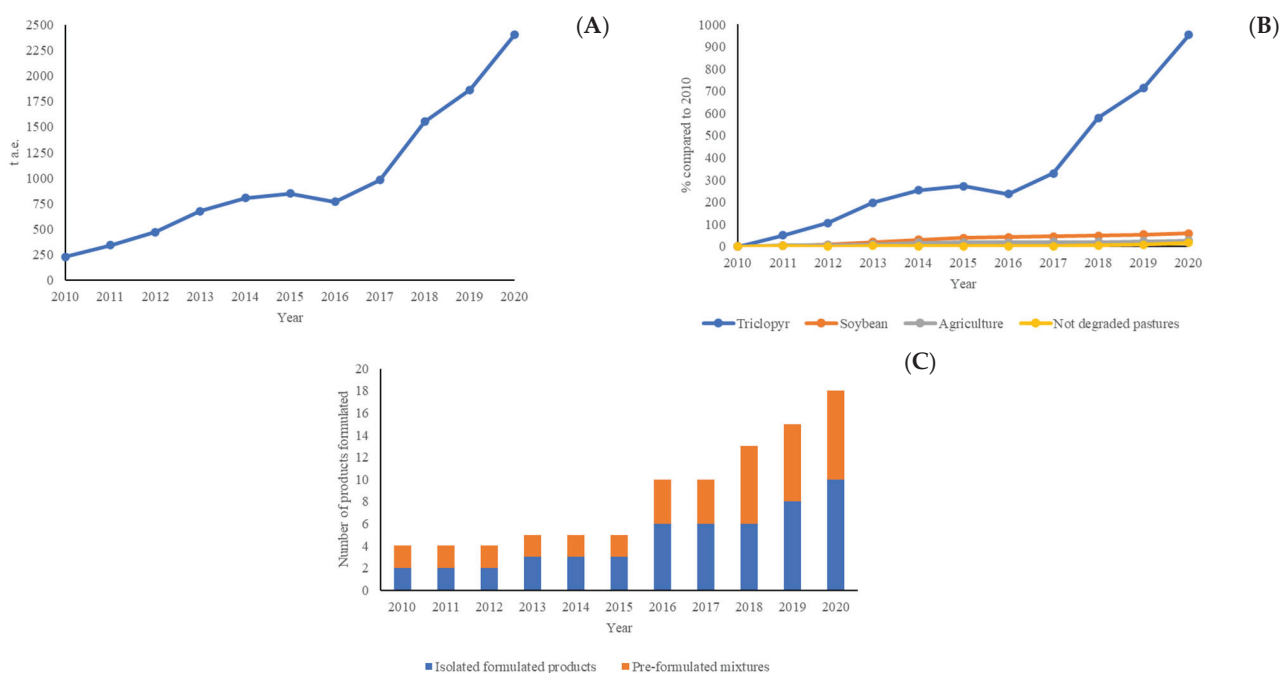


Figure 2. Sales of triclopyr (tons of a.e.) from 2010 to 2020 (A). Evolution of the commercialization of herbicide in relation to the year 2010, compared to the evolution of the soybean area (IBGE), the area of agriculture intended for harvest (temporary and permanent) (IBGE), and the area cultivated with pastures showing no degradation (LAPIG/UFG) (B). Number of formulated products based on triclopyr marketed in Brazil (C) (period from 2010 to 2020).

Haloxypop-methyl and clethodim are herbicides used for post-emergence control of grasses, being selective for broadleaf crops such as soybean [68]. Both herbicides inhibit the enzyme acetyl-CoA carboxylase (ACCase), blocking lipid synthesis and, consequently, the formation of cell walls [69]. These two graminicides had increases in sales in Brazil between 2010 and 2020 (Figure 3A). In 2010, 111.3 tons of haloxypop-methyl and 244.5 tons of clethodim (active ingredient/acid equivalent) were sold. By 2020, these amounts rose to 1109.8 tons of haloxypop-methyl and 6779.6 tons of clethodim, representing an 896.9% increase in haloxypop-methyl consumption and a 2672.8% increase for clethodim (Figure 3B). These increases far exceed the growth in agricultural area (24.3%) and soybean-planted area (59.4%) during the same period.

Among the main reasons for the sharp increase in the consumption of haloxypop-methyl and clethodim herbicides are: (1) a significant rise in grain-growing areas infested with glyphosate-resistant biotypes of *Digitaria insularis* [26]. This situation created the need for supplementary control of this species, both in pre-sowing burndown operations and in post-emergence soybean management, substantially increasing the demand for these herbicides. More recently, another grass species resistant to glyphosate that could contribute to the increased use of haloxypop-methyl and clethodim is *Eleusine indica*. However, this trend has not been observed with the same intensity, as multiple-resistant biotypes (EPSPS

and ACCase) of this species have spread, limiting the effectiveness of these herbicides [70]; and (2) the increasing challenge posed by volunteer glyphosate-resistant corn plants. With the growing adoption of glyphosate-resistant corn hybrids in Brazil, controlling volunteer corn plants, resulting from mechanical harvesting failures, has become more problematic. This created the need to introduce new herbicides for controlling these plants before soybean sowing or even in established soybean fields. Among the effective herbicides for this purpose are ACCase inhibitors, such as haloxyfop-methyl and clethodim [71].

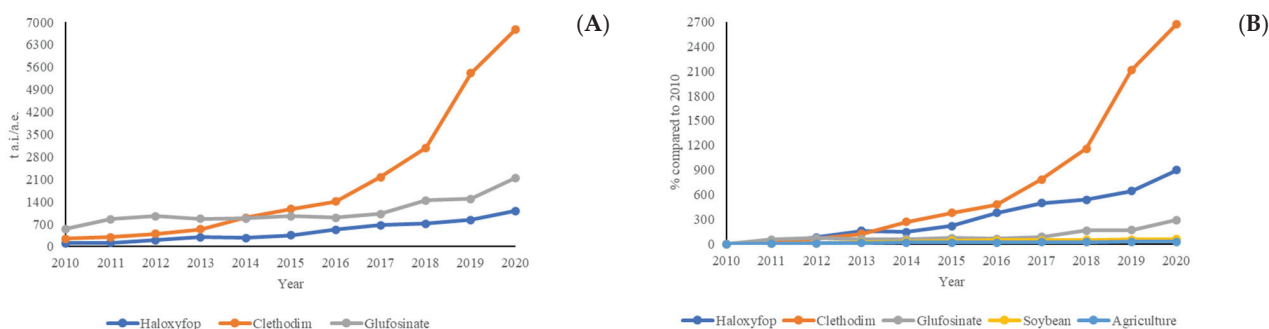


Figure 3. Sales of haloxyfop-methyl, clethodim, and glufosinate (tons of a.i./a.e.) (A). Evolution of the commercialization of herbicides in relation to the year 2010, compared to the evolution of the soybean area (IBGE) and the area of agriculture intended for harvest (temporary and permanent) (IBGE) (B). Sales data for haloxyfop-methyl are presented in effective ingredients.

The increased availability of haloxyfop-methyl and especially clethodim products may have also contributed to the rise in herbicide sales. In 2010, only one formulated product containing the isolated active ingredient haloxyfop-methyl was available in the Brazilian market. By 2020, this number had grown to nine products, including seven with haloxyfop-methyl as a single active ingredient and two pre-formulated mixtures (Figure 4A), representing an 800% increase in haloxyfop-methyl-based product availability over 11 years. For clethodim, the increase in formulated products during the same period was 600%, rising from two products in 2010 (one isolated and one pre-formulated mixture) to 14 products in 2020 (11 isolated and three pre-formulated mixtures) (Figure 4B).

The higher increase in clethodim consumption compared to haloxyfop-methyl may be related to the lower cost per hectare of clethodim application. It is important to note that the rise in glyphosate-resistant *Digitaria insularis* biotypes and volunteer corn plants (Roundup Ready™—RR™) was a key driver encouraging pesticide manufacturers and importers to register and market generic products based on haloxyfop-methyl and clethodim in Brazil, as these resistance cases created substantial demand for this group of herbicides. In summary, the increased availability of formulated products can be seen as a response to the worsening weed resistance to glyphosate in Brazil.

Three key factors can be identified as predominant drivers of the increase in the commercialization of the herbicide glufosinate in Brazil (Figures 3 and 4C): (1) increase in areas infested with glyphosate-resistant weeds, such as *Conyza* spp., *Digitaria insularis*, *Eleusine indica*, and *Amaranthus* spp. [72]. This is the most important factor driving the rise in glufosinate sales in Brazil; (2) introduction of transgenic soybean, corn, and cotton cultivars resistant to post-emergence applications of glufosinate in the Brazilian market; and (3) growth in the availability of glufosinate-based formulated products, especially after 2017 (one formulated product in 2010; nine in 2020). This increase is largely due to the sharp rise in glyphosate-resistant weeds in Brazil's grain crops, which has spurred companies' interest in marketing this active ingredient. It is also important to note that the ban on paraquat use in Brazil had a significant impact on glufosinate sales. Glufosinate shares some similar agronomic characteristics with paraquat, such as a broad spectrum of control, contact action, and the absence of residual soil activity [73]. However, the impact of this ban became more evident only in the later years of the analyzed period.

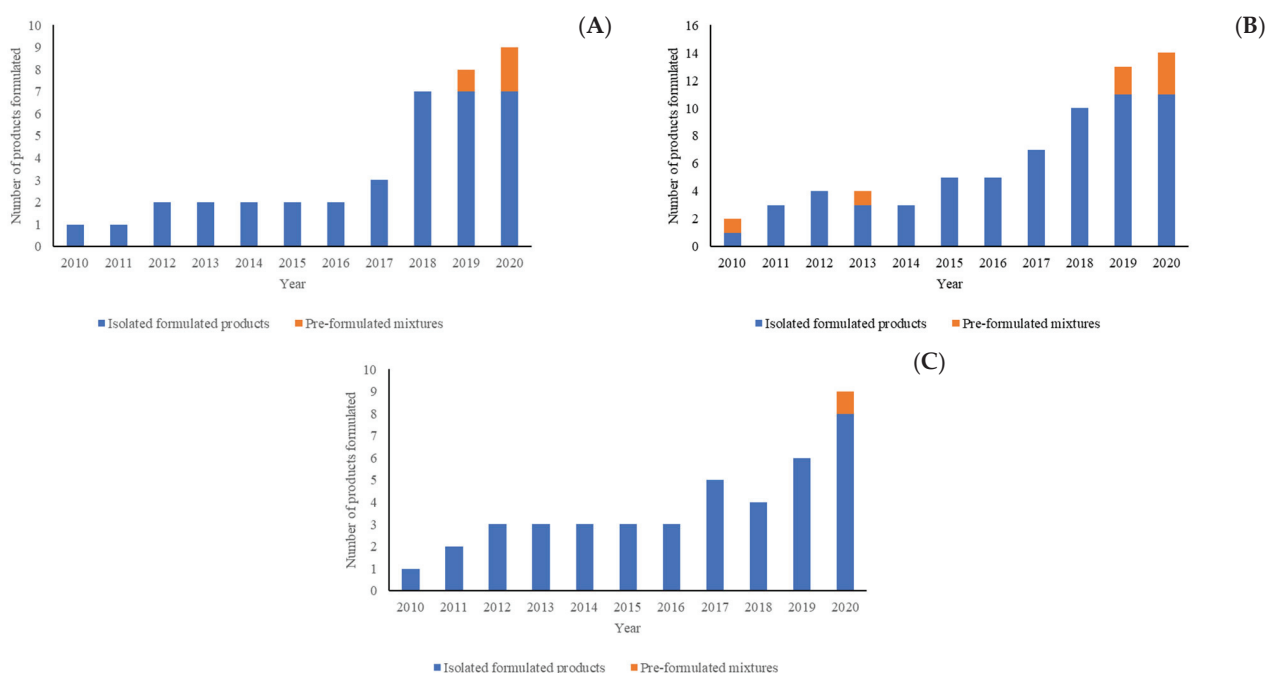


Figure 4. Number of formulated products based on haloxyfop-methyl (A), clethodim (B), and glufosinate (C) marketed in Brazil, subdivided by products containing only the active ingredient (isolated) or pre-formulated mixtures containing the respective herbicides (period from 2010 to 2020).

In the context of glyphosate-resistant weeds, the use of herbicides with residual soil activity has re-emerged in recent years in grain production areas. Looking ahead, there are expectations for the increased adoption of these herbicides in agricultural production systems, as they can control weed populations in the pre-emergence stage of crops [74]. Figures 5 and 6 show the sales data (tons of active ingredient and percentage growth) and the number of formulated products for the herbicides diclosulam and flumioxazin, which experienced sales growth between 2010 and 2020. Although flumioxazin had higher sales volumes, comparing the application rates per hectare for the crops registered for diclosulam suggests that diclosulam usage also increased substantially and is comparable to flumioxazin (Figure 5A). Moreover, considering only the percentage increase in sales relative to the first year of analysis (2010), both diclosulam and flumioxazin saw growth, with increases exceeding 520.0% (Figure 5B).

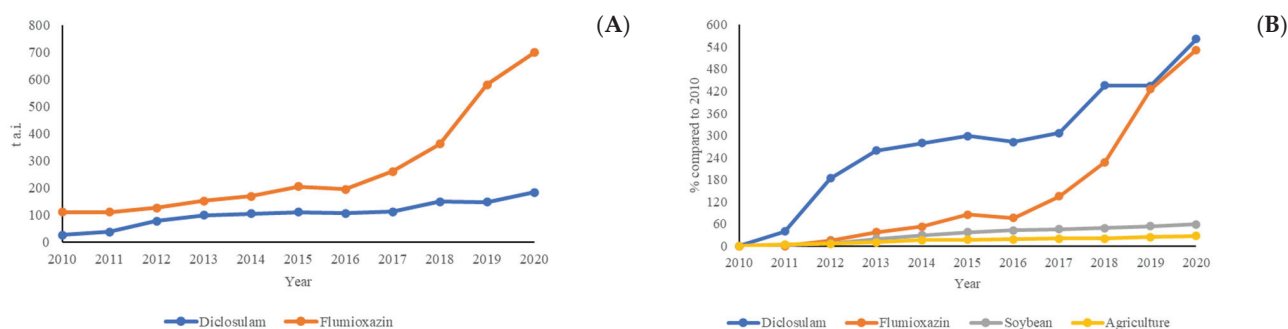


Figure 5. Sales of diclosulam and flumioxazin (tons of a.i.) (A). Evolution of herbicide sales compared to the year 2010, alongside the evolution of soybean area (IBGE) and the area of agriculture designated for harvest (temporary and permanent) (IBGE) (B).

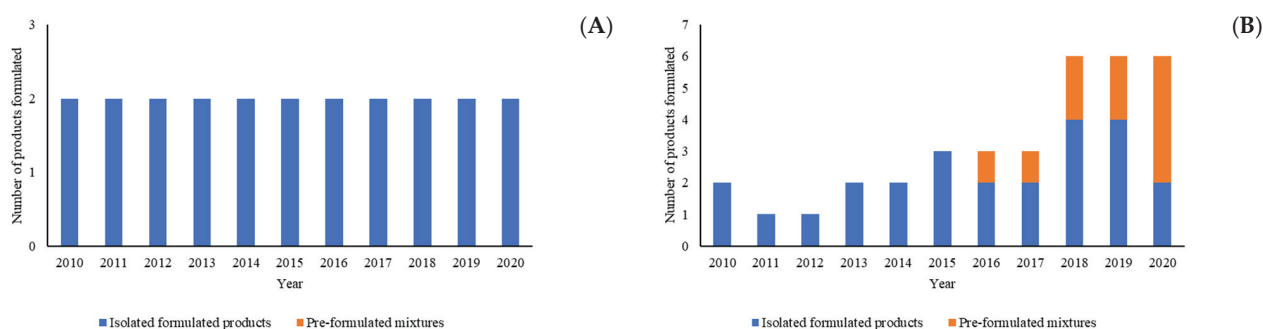


Figure 6. Number of formulated products based on diclosulam (A) and flumioxazin (B) marketed in Brazil, subdivided by products containing only the active ingredient (isolated) or pre-formulated mixtures containing the respective herbicides (period from 2010 to 2020).

Several factors can explain the increase in the commercialization of diclosulam and flumioxazin in the Brazilian market, among which the following stand out: (1) increased adoption of herbicides used in soybean pre-emergence applications, as certain products containing these active ingredients rank among the most widely used for this purpose [75]; (2) greater occurrence of glyphosate-resistant weeds, including *Conyza* spp. and the *Amaranthus* spp. complex (*A. hybridus* and *A. palmeri*), which diclosulam and flumioxazin effectively control in pre-emergence [60]; and (3) increased use of herbicides with residual soil activity, such as diclosulam and flumioxazin, in pre-sowing burndown applications for soybean crops.

Regarding the evolution of formulated products containing diclosulam and flumioxazin in the Brazilian agrochemical market, distinct behaviors can be observed depending on the active ingredient (Figure 6). For diclosulam, only two formulated products containing this active ingredient, one registered for sugarcane and another for soybean, were marketed during the period analyzed in this study (Figure 6A). Given the herbicide's high efficacy in controlling the main weeds infesting the crops for which diclosulam is registered [76], future prospects suggest a potential increase in the number of formulated products based on this active ingredient.

For flumioxazin, the number of formulated products marketed in Brazil increased, particularly from 2015 onward (Figure 6B). This reflects one of the unique characteristics of flumioxazin's weed control action: unlike other herbicides, flumioxazin exhibits residual soil activity (pre-emergence control), offering the added benefit of effective weed control in early post-emergence stages through contact action [77,78]. This is especially true when flumioxazin is used in combination with other herbicides. Additionally, due to its lower acquisition cost compared to other pre-emergent herbicides, along with these agronomic advantages, the number of formulated products containing flumioxazin has increased in recent years.

Despite the issues of weed resistance and tolerance discussed throughout this manuscript, data presented in Table 2 show that the commercialization of glyphosate in Brazil increased from 101,385 tons of active ingredient in 2010 to 209,345 tons in 2020, representing a 106.5% increase over the period. Furthermore, in 2010, glyphosate sales accounted for 64.37% of total herbicide sales in Brazil, whereas in 2020 this percentage dropped to 58.26%, indicating that other herbicides experienced higher growth rates in sales compared to glyphosate. This shift may reflect the widespread occurrence of glyphosate-resistant biotypes in agricultural areas across the country. Notably, glyphosate accounted for 34.33% of all pesticide sales in Brazil in 2010, a scenario that remained virtually unchanged in 2020 (34.31%). Several factors may explain the increased use of glyphosate in Brazil, including: (1) expansion of no-till farming; (2) greater adoption of GMO cultivars resistant to glyphosate, such as soybean, corn, and cotton; (3) increased use of cover crops (e.g., *Brachiaria* and *Panicum*), either in monoculture or intercropped systems, where glyphosate is the main tool for pre-sowing burndown [79]; (4) additive or synergistic effect when mixed with other herbicides with different modes of action, enhancing weed control [80]; and

(5) increased availability of glyphosate-based products. In 2010, 36 glyphosate-formulated products were sold in Brazil, including 35 single-ingredient products and only one pre-mix. By 2020, this number rose to 47 products, a 30.6% increase, with 45 single-ingredient products and two pre-mixes (Table 2).

Table 2. Information on glyphosate commercialization in Brazil in 2010 and 2020.

Information	Year	
	2010	2020
Glyphosate commercialization (tons of active ingredients)	101,385	209,345
% of total herbicide commercialization	64.37	58.26
% of total pesticide commercialization	34.33	34.31
Number of Formulated products	36	47
Number of isolated formulated products	35	45
Number of pre-formulated mixtures with other active ingredients	1	2
Salt formulation	% of total glyphosate sales	
Isopropylamine	51.59	12.55
Ammonium	21.49	27.06
Potassium	26.92	35.88
Dimethylammonium	0.00	0.75
Diammonium	0.00	9.26
[Isopropylamine + potassium]	0.00	14.50

The type of salt formed from glyphosate also changed between 2010 and 2020. Chemically, glyphosate is formulated as ammonium, diammonium, dimethylammonium, potassium, or isopropylamine salts [81]. In 2010, isopropylamine salt accounted for 51.59% of glyphosate sales in Brazil. However, by 2020, its share dropped to 12.55%, with potassium salt becoming the most sold, representing 35.88% of total glyphosate sales (Table 2). Changes in the type of glyphosate salt used in commercial products can affect the speed of weed control [22] and influence environmental factors after application, such as absorption speed and the impact of rainfall following application (rainfastness).

The data indicate disproportionate growth in the sale of herbicides used to manage glyphosate-resistant weeds, including both pre- and post-emergence products. This suggests that the resistance of weeds to glyphosate is a primary factor driving increased herbicide consumption. As a result, the quantitative data clearly highlight the negative impacts of glyphosate resistance, leading to agronomic and economic losses and significantly increasing the need for additional herbicides.

Despite the spread of resistant biotypes, glyphosate has not been widely replaced by other herbicides. Instead, it is often supplemented with additional molecules [82], as discussed in this study, contributing to the rising consumption of herbicides in Brazil. This increase in herbicide use is primarily due to a greater number of active ingredients applied per season, not higher doses. It is important to note that herbicide selectivity for crops is based on dose limits [83], since exceeding these limits can cause significant yield losses. Before resistance became widespread, glyphosate isolated was considered sufficient to control weeds across many agricultural areas [84]. Today, however, most soybean fields require two, three, or more active ingredients to complement glyphosate's action, both before sowing (burndown) and during the crop cycle (pre- and post-emergence).

Given this scenario, it is essential to implement integrated weed management strategies, including preventive control (e.g., cleaning machinery and acquiring certified, weed-free seeds), and cultural control (e.g., crop rotation, no-till farming with surface mulch, narrower row spacing, selecting fast-growing cultivars, and using high-vigor seeds). Additionally, whenever possible, herbicide rotation with different modes of action should be practiced, along with the use of sprayers equipped with weed-detecting sensors for post-emergence applications. Research investments aimed at identifying biological control agents to develop bioherbicides, as well as improving physical weed control methods (e.g.,

robotics, laser beams, electromagnetic waves, or boiling water with sealing foam), are also strategic and sustainable approaches. These combined efforts are crucial to preventing a future collapse in chemical weed control effectiveness and avoiding unsustainable herbicide loads in agricultural environments.

4. Conclusions

The commercialization of herbicides in Brazil saw an increase between 2010 and 2020. Some herbicides, such as clethodim, haloxyfop-methyl, triclopyr, glufosinate, 2,4-D, diclosulam, and flumioxazin, experienced increases in sales of 2672.8%, 896.9%, 953.5%, 290.2%, 233.8%, 561.3%, and 531.6%, respectively, far exceeding the growth rate of Brazil's agricultural area. Several factors may have contributed to this substantial rise in herbicide consumption; however, the primary cause was the worsening cases of glyphosate-resistant weed biotypes in Brazilian agricultural areas, particularly among species such as *Conyza* spp., *Amaranthus* spp., *Digitaria insularis*, and *Eleusine indica*. This situation created the need to supplement glyphosate with other herbicides to achieve effective chemical control of these species.

The data highlight that weed resistance to glyphosate has driven a substantial increase in the commercialization of herbicides with different profiles for both pre- and post-emergence applications, resulting in agronomic and economic impacts. This scenario led to the need for glyphosate to be supplemented with other molecules rather than replaced, contributing to an increase in the number of active ingredients used per season. This situation underscores the importance of integrated practices, such as rotating herbicide modes of action and implementing preventive and cultural controls, to ensure the effectiveness of chemical control and reduce dependency on herbicides. Investments in bioherbicides, nanotechnology related to herbicide formulation, and physical methods such as robotics and laser emissions are becoming essential to tackle future challenges and offer innovative and sustainable alternatives in weed management.

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Article

Assessment of Fumonisin, Deoxynivalenol, and Zearalenone Levels and the Occurrence of Mycotoxigenic *Fusarium* Species in Cereal Grains from Muscat, Sultanate of Oman

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Abstract: Mycotoxin contamination in agricultural goods is a major global problem due to its negative impact on human and animal health. The principal mycotoxin producers are fungal species from the genera *Fusarium*, *Aspergillus*, *Alternaria*, and *Penicillium*. The toxigenic fungal species produce the mycotoxins as secondary metabolites when they invade agricultural commodities during crop cultivation in the field (preharvest) or after harvesting or during transport and storage. This study was designed to investigate the levels of *Fusarium* mycotoxins, viz., fumonisin (FUM), zearalenone (ZEN), and deoxynivalenol (DON) in cereal grain samples collected from Muscat, Sultanate of Oman during 2023–24. A total of 90 cereal grain (wheat, corn, rice, barley) samples from local markets at Muscat, the Plant Quarantine Department, Oman, and Oman Flour Mills Company were analyzed using competitive enzyme immunoassay kits. Furthermore, *Fusarium* spp. associated with the contaminated grain samples were isolated, and their mycotoxin-producing potential was assessed. The results indicated that FUM, ZEN, and DON levels were below the detection limit (LOD) in 81%, 97%, and 44% of the samples, respectively. Two out of fifteen corn samples and one out of thirty-seven wheat samples tested exceeded the maximum permissible limit for FUM and ZEN, respectively, as set by the European Commission. A total of 19 *Fusarium* spp. associated with the contaminated grain samples were isolated and identified through molecular techniques. Sixteen isolates of *F. verticillioides*, one isolate of *F. thapsinum*, and two new *Fusarium* species were identified based on nuclear ribosomal DNA internal transcribed spacer and elongation factor 1- α sequences. Two isolates of *F. verticillioides* (FQD-1 and FQD-20) produced FUM levels exceeding 2000 $\mu\text{g kg}^{-1}$. The maximum ZEN concentration was observed in *F. verticillioides* FQD-20 (9.2 $\mu\text{g kg}^{-1}$), followed by *F. verticillioides* FQD-2 (2.8 $\mu\text{g kg}^{-1}$) and *Fusarium* sp. FOFMC-26 (2.5 $\mu\text{g kg}^{-1}$). All tested *Fusarium* strains produced DON, with levels ranging from 25.6 to 213 $\mu\text{g kg}^{-1}$, with *F. thapsinum* FQD-4 producing the highest level (213 $\mu\text{g kg}^{-1}$). To our knowledge, this is the first report on the occurrence of *Fusarium* mycotoxins and mycotoxigenic *Fusarium* spp. in food commodities in Oman.

Keywords: cereals; enzyme-linked immunosorbent assay; food safety; *Fusarium* mycotoxins; molecular characterization; toxigenic *Fusarium*

1. Introduction

Food security is a serious issue among the global communities as the world population anticipated to expand to 9.7 billion by 2050 [1]. Threats to food security include limitations

in the supply of nutritious and safe foods to consumers [2,3]. Mold contamination in agricultural commodities is considered as a major food safety hazard, as several mold species secrete harmful toxic secondary metabolites known as “mycotoxins” on various food matrices. Reports indicate that 5–10% of agricultural products worldwide are spoiled by mold contamination, rendering them unfit for human or animal consumption. Several mold species from the genera *Fusarium*, *Aspergillus*, *Alternaria*, and *Penicillium* produce mycotoxins on the substrates during their growth under favorable conditions [4]. This contamination can occur at various stages, including crop cultivation in the field, harvest, storage, and processing [5]. Consuming food that is contaminated with mycotoxins might lead to mycotoxicosis and food poisoning, potentially causing death in both humans and animals [6]. There are around 300 different mycotoxins, but only about 20 are known to cause toxic effects on humans when consumed through contaminated food. These include aflatoxin, sterigmatocystin, ochratoxin (A, B, C), trichothecenes (deoxynivalenol, diacetoxyscirpenol, T2 toxin, HT-2 toxin, nivalenol), fumonisin (B1, B2), moniliformin, zearalenone, cyclopiazonic acid, patulin, alternariol monomethyl ether, alternariol, tenuazonic acid, and citrinin [7]. Among them, fumonisins, aflatoxins, zearalenone, and deoxynivalenol are frequently found in cereal grains. Each mycotoxin is associated with specific health risks in humans and animals. Aflatoxins are considered the most carcinogenic mycotoxin and are classified in Group I. Fumonisin and ochratoxins are classified in Group 2B, while trichothecenes and zearalenone are categorized as non-carcinogenic and placed in Group 3 [8]. These mycotoxins are exceptionally stable compounds that can withstand high temperatures, storage, and processing conditions [9]. For example, DON remains stable at 120 °C, showing moderate stability at 180 °C and partial stability at 210 °C [10]. ZEN can be inactivated after 60 min at 175 °C [11], whereas fumonisins remain stable even at temperatures as high as 250 °C [12].

Fusarium spp. produce three major classes of mycotoxins as secondary metabolites: fumonisins, trichothecenes, and zearalenone [13]. Fumonisin (FUM) contamination is common in maize and maize-based products. Fumonisin is primarily produced by *F. verticillioides* and *F. proliferatum* as secondary metabolites [14,15]. Other *Fusarium* species such as *F. oxysporum*, *F. napiforme*, *F. nygamai*, *F. anthophilum*, and *F. dlamini* are also known to produce fumonisins [16,17]. About 15 types of fumonisin (fumonisin A, B, C, and P) have been characterized [18]. The primary food contaminants are fumonisin B1 (FB1), fumonisin B2 (FB2), and fumonisin B3 (FB3), with FB1 being the most dangerous [19]. *F. verticillioides* is an important pathogen in corn, causing seedling blight, ear rot, seed rot, and stalk rot. The pathogen produces fumonisins mainly during the pre-harvesting stage, and under improper storage conditions, the pathogen continues to produce the toxin during the post-harvesting stage [20]. Besides corn, fumonisins have been detected in wheat, barley, rye, oat, rice, and other millets [17,21]. Other food products reported to contain FB1 include dried figs, garlic, asparagus, beers, and milk [17]. Fumonisin has been connected with esophageal cancer [17] and harmful effects on the liver and kidneys in animals [22]. Fumonisin consumption has been associated with pulmonary edema syndrome in pigs [23], leukoencephalomalacia in horses [24], hepatotoxicity and nephrotoxicity in rats [25], and apoptosis in several different types of animal cells [26].

Zearalenone (ZEN; F-2 toxin; $C_{18}H_{22}O_5$) is a non-steroidal estrogenic compound primarily produced by *F. graminearum*, *F. oxysporum*, *F. verticillioides*, *F. culmorum*, *F. cerealis*, *F. semitectum*, *F. equiseti*, *F. acuminatum*, *F. sporotrichioides*, and *F. crookwellense* [27]. Maize, wheat, barley, oats, rice, rye, and sorghum are highly susceptible to ZEN contamination [28]. ZEN is a xenoestrogen biosynthesized via polyketide pathway [27]. The chemical structure of ZEN mimics natural estrogens like 17 β -estradiol, allowing it to bind to estrogen receptor sites and disrupt hormonal balance, leading to various reproductive system diseases [29]. Furthermore, ZEN is rapidly absorbed in the body of mammals and metabolized into highly toxic compounds [30]. ZEN exposure has been proven to diminish the progesterone and

serum testosterone levels in the bloodstream, resulting in sterility and decreased conception rates in animals such as rats, pigs, and cows [31,32].

Deoxynivalenol (DON; Vomitoxin; $C_{15}H_{20}O_6$) is a trichothecene mycotoxin produced by many *Fusarium* species such as *F. graminearum*, *F. oxysporum*, *F. avenae*, *F. asiaticum*, and *F. culmorum* [33,34]. Corn, wheat, barley, rye, and oats are commonly contaminated by DON following infection by the toxigenic fungi in the field and during storage [35,36]. DON is a highly stable mycotoxin in both storage and processing [37,38]. Acute poisoning from DON causes emesis, while chronic low-dose exposure leads to anorexia, growth retardation, immunotoxicity, and reproduction impairment in experimental animal models [39–41]. In addition, DON is phytotoxic and is regarded as a virulence factor in fungal pathogenesis [42–44]. In many cases, these mycotoxins can co-exist in agricultural commodities, leading to synergistic, additive, or antagonistic toxic effect on the host [45].

According to the European Commission regulatory standards, the maximum tolerable limits of fumonisins (FB1 + FB2) in unprocessed corn; deoxynivalenol in unprocessed durum wheat, oats, and maize; and zearalenone in unprocessed corn are 2000, 1750, and 200 $\mu\text{g kg}^{-1}$, respectively [46]. Oman sources most of its cereal grains from international markets [47]. Though there are a few reports on the occurrence of aflatoxins in food commodities [48–50], studies on the level of *Fusarium* toxins in imported and marketed agricultural products in Oman is limited. This study was conducted to quantify the levels of fumonisin, deoxynivalenol, and zearalenone in cereal grains (wheat, corn, rice, and barley) collected from Muscat, Oman. The occurrence of toxigenic strains of *Fusarium* in the mycotoxin-contaminated samples was also investigated.

2. Materials and Methods

2.1. Sample Collection

Ninety cereal grain samples consisting of wheat (37 samples), corn (15 samples), rice (5 samples), and barley (33 samples) (100 g to 1 kg) were collected from the local markets in Muscat, the Plant Quarantine Department of the Ministry of Agriculture, Fisheries and Water Resources (MAFWR), Oman, and Oman Flour Mills Company (S.A.O.G), Muscat in 2023 and 2024. The collected samples were analyzed for grain contamination with FUM, ZEN, and DON.

2.2. Mycotoxin Estimation

A quantitative analysis of *Fusarium* mycotoxins in the cereal grain samples was performed using RIDASCREEN® Fumonisin ECO (Art. No. R3411), RIDASCREEN® Zearalenon (Art. No. R1401), and RIDASCREEN® DON (Art. No. R5906) competitive enzyme immunoassay kits (R-Biopharm AG, Darmstadt, Germany), following the manufacturer's instructions. These kits had detection limits of 30 $\mu\text{g kg}^{-1}$ for Fumonisin and 1.75 $\mu\text{g kg}^{-1}$ for Zearalenone and 18.5 $\mu\text{g kg}^{-1}$ for DON. The software RIDASOFT Win.NET Food & Feed Version 1.5.2 (Art. No. Z9996FF; R-Biopharm AG, Darmstadt, Germany) was used for determination of mycotoxin levels.

2.3. Isolation of *Fusarium* spp.

The cereal grains (wheat, corn, rice, and barley) were surface-sterilized with 1% NaOCl for 2 min. To eliminate NaOCl residues, the samples were washed three times with sterilized distilled water (SDW) before being plated on a potato dextrose agar (PDA) medium (Oxoid Ltd., Basingstoke, UK). The plates were then incubated for 3–5 days at 27 °C. Pure cultures of the fungi were obtained by using the hyphal tip isolation method.

2.4. DNA Extraction, Amplification, and Sequencing

Lee and Taylor's [51] method was followed to recover the DNA from the fungal mycelium. The fungal mycelium (~80 mg) was taken in a sterile 2 mL centrifuge tube from a 7-day-old PDA culture with a sterile scalpel and ground in the presence of acid-washed

sand and suspended in 600 µL extraction buffer (50 mM Tris-HCl pH 7.6, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol). The sample was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, *v/v*; Sigma-Aldrich, St. Louis, MO, USA). DNA was precipitated with 1/10 volume of 3 M sodium acetate and 0.6 volume of isopropanol, washed with 70% ethanol, and resuspended in 50 µL SDW. A NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the quality and amount of isolated DNA.

The primer combinations ITS5/ITS4 [52] and EF-1 α -F/EF-1 α -R [53] were used to amplify two DNA regions: internal transcribed spacer (ITS) and translation elongation factor (EF-1 α) (Table 1). The reaction mixture (25 µL) consisted of 1 µL of DNA template (100 ng), 1 µL of each primer (10 µmol), a puReTaq Ready-to-Go PCR bead (GE Healthcare, Buckinghamshire, UK), and 22 µL of nuclease free water [54]. For ITS amplification, the PCR conditions described by Hussain et al. [55] were used. At Macrogen Inc. (Seoul, Republic of Korea), the PCR products were purified and subsequently sequenced in both directions using the same primers.

Table 1. Primers used for identification of fungi in this study.

Gene	Primer	Direction	Sequence (5'→3')
ITS	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG
	ITS4	Reverse	TCCTCCGCTTATTGATATGC
EF-1 α	EF-1 α -F	Forward	ATGGGTAAGGAAGACAAGAC
	EF-1 α -R	Reverse	GGAAGTACCAGTGATCATGTT

2.5. Sequence Alignment and Phylogenetic Analyses

BioEdit v. 7.0.9 was used to align and transform the forward and reverse primer readings of the ITS and EF-1 α regions into consensus sequences [56]. Sequence similarity was checked against the GenBank sequences using BLAST tools (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (accessed on 3 September 2024). The most similar species in BLAST results were *F. verticillioides*, *F. thapsinum*, and *F. chlamydosporum*. It is obvious from the literature that both *F. verticillioides* and *F. thapsinum* belonged to the *F. fujikuroi* species complex [57]. Therefore, we constructed a combined ITS-EF-1 α dataset comprised of the *F. fujikuroi* species complex and the *F. chlamydosporum* species complex along with representative species from other species complexes [58]. The dataset consisted of 52 specimens, including *Fusicolla aquaeductuum* (CBS 734.79) as an outgroup taxon. The data matrix was aligned using Mafft v. 7 (<https://mafft.cbrc.jp/alignment/server/>) (accessed on 3 September 2024) [59]. A maximum likelihood (ML) method was used for the phylogenetic analyses, with the ML phylogeny generated using RAxML-HPG BlackBox on the Cipres Science Gateway [60,61]. According to jModelTest2, the best model (GTR + F + I + G4) was chosen [62]. In total, 1000 bootstrap repeats were used to evaluate branch support for the ML phylogeny, and a bootstrap (BT) percentage of >50 was deemed significant. The phylogenetic tree was visualized using FigTree v.1.4.2 [63] and annotated with Adobe Illustrator CC2019.

2.6. Mycotoxigenic Potential of *Fusarium* spp.

The mycotoxin-producing potential of *Fusarium* strains on the maize grain substrate was assessed following a method described by Shi et al. [64]. Briefly, 25 mL of distilled water was added to 50 g of mycotoxin-free maize and left overnight. The medium was then sterilized by autoclaving at 121 °C for 20 min. A 6 mm diameter agar disk cut from a 7-day-old PDA culture of the fungus was transferred to the sterilized maize grain medium and incubated at 25 °C for 21 days. After that, the medium containing a fungal mycelial mat was dried in an oven at 40–50 °C until constant weight was achieved, then ground into powders and analyzed for mycotoxins (FUM, DON, ZEN) by using R-Biopharm RIDASCREEN competitive enzyme immunoassay kits as described earlier. The uninoculated maize

grain medium prepared in the same manner served as the control. Each treatment was replicated thrice.

3. Results

3.1. Occurrence of Fumonisin

Fumonisin levels in 73 out of 90 grain samples (81%) were below the detection limit (Table 2). Twelve corn samples, three wheat samples, and two barley samples tested positive for fumonisin. In general, corn samples exhibited higher levels of fumonisin, with two out of the fifteen tested samples exceeding the EC's maximum tolerance level of 2000 $\mu\text{g kg}^{-1}$ for fumonisin. All five rice samples tested were below the detection limit for fumonisin.

Table 2. The levels of fumonisin in cereal grain samples collected from local markets, Oman flour mills company, and quarantine department, in Muscat, Oman.

Food Items	Analyzed Samples	Sample Count and Range of Fumonisin Concentrations ($\mu\text{g kg}^{-1}$)			
		<LOD (<30)	31–1000	1001–2000	>2000
Corn	15	3	9 (38.7–954.3)	1 (1096.2)	2 (2011.3–2808.5)
Wheat	37	34	3 (34.9–95.9)	0	0
Rice	5	5	0	0	0
Barley	33	31	2 (45.1–712.9)	0	0
Total	90	73	14	1	2

3.2. Occurrence of Zearalenone

The results indicated that 87 out of 90 grain samples tested (97%) were below the detection limit (Table 3). One corn sample, one barley sample, and one wheat sample tested positive for zearalenone, with the wheat sample exceeding the EC's maximum tolerance level of 200 $\mu\text{g kg}^{-1}$ for zearalenone. All five rice samples were below the detection level for zearalenone.

Table 3. The levels of zearalenone in cereal grain samples collected from local markets, Oman flour mills company, and quarantine department, in Muscat, Oman.

Food Items	Analyzed Samples	Sample Count and Range of Zearalenone Concentrations ($\mu\text{g kg}^{-1}$)			
		<LOD (<1.75)	1.76–100	101–200	>200
Corn	15	14	1 (2.7)	0	0
Wheat	37	36	0	0	1 (293.4)
Rice	5	5	0	0	0
Barley	33	32	1 (40.7)	0	0
Total	90	87	2	0	1

3.3. Occurrence of DON

Out of ninety cereal grain samples tested, forty samples (44%) were below the detection level for DON, and none exceeded the EC's maximum tolerance level of 1750 $\mu\text{g kg}^{-1}$ for unprocessed durum wheat, oats, or maize (Table 4). Nine of the fifteen corn samples had DON levels ranging from 21 to 398 $\mu\text{g kg}^{-1}$, with one sample reaching 519 $\mu\text{g kg}^{-1}$. Among the thirty-seven wheat samples, nineteen had DON levels ranging from 22 to 311 $\mu\text{g kg}^{-1}$, with one sample reaching 559 $\mu\text{g kg}^{-1}$. The DON levels in three out of five rice samples ranged from 20 to 56 $\mu\text{g kg}^{-1}$, while seventeen out of thirty-three barley samples had levels between 19 and 67 $\mu\text{g kg}^{-1}$, respectively.

Table 4. The levels of deoxynivalenol (DON) in cereal grain samples collected from local markets, Oman flour mills company, and quarantine department, in Muscat, Oman.

Food Items	Analyzed Samples	Sample Count and Range of Deoxynivalenol Concentrations ($\mu\text{g kg}^{-1}$)				
		<LOD (<18.5)	18.6–500	501–1000	1001–1500	>1500
Corn	15	5	9 (20.7–398.3)	1 (519.3)	0	0
Wheat	37	17	19 (22.4–311.5)	1 (559.4)	0	0
Rice	5	2	3 (20.1–56.0)	0	0	0
Barley	33	16	17 (19.2–66.7)	0	0	0
Total	90	40	48	2	0	0

3.4. Isolation of *Fusarium* spp. from Cereal Grains

A total of 19 *Fusarium* species associated with the contaminated cereal grain samples were isolated on the PDA medium. Initial BLAST analysis of the ITS sequences confirmed that the isolates belonged to the *Fusarium* genus. Further identification of species within the *Fusarium* genus was conducted using combined ITS and EF-1 α -sequence alignment.

3.5. Phylogenetic Analysis

The final ITS-EF-1 α dataset was 2104 characters long, including 1557 constant sites, 390 parsimony informative sites, and 157 uninformative sites. The ML phylogeny is presented in Figure 1, where the species of *Fusarium* recovered in two clades. Clade-I corresponding to the *F. fujikuroi* species complex (FFSC) and Clade-II to the *F. chlamydosporum* species complex (FCSC), *F. tricinctum* species complex (FTSC), *F. citricola* species complex (FCCSC), and *F. incarnatum-equiseti* species complex (FIESC), respectively. There were sixteen isolates of *F. verticillioides* and one isolate of *F. thapsinum* (FQD-4) recovered in this study. Both *F. verticillioides* and *F. thapsinum* fall in FFSC. The two isolates (FOFMC-26, FOFMC-27), representing an undescribed species in *Fusarium*, recovered with an independent lineage in Clade-II. The GenBank accession numbers of the ITS and EF-1 α gene sequences of the *Fusarium* spp. isolated in this study are given in Table 5.

Table 5. *Fusarium* species isolated from mycotoxin-contaminated cereal grains and GenBank accession numbers for the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (EF-1 α) sequences.

Isolate	Source	Identified Fungus	GenBank Accessions	
			ITS	EF-1 α
FQD-1	Wheat	<i>Fusarium verticillioides</i>	PQ325649	PQ349965
FQD-2	Wheat	<i>Fusarium verticillioides</i>	PQ325650	PQ349966
FQD-3	Corn	<i>Fusarium verticillioides</i>	PQ325651	PQ349967
FQD-9	Barley	<i>Fusarium verticillioides</i>	PQ325652	PQ362702
FQD-10	Barley	<i>Fusarium verticillioides</i>	PQ325653	PQ604778
FQD-11	Barley	<i>Fusarium verticillioides</i>	PQ325654	PQ369625
FQD-12	Barley	<i>Fusarium verticillioides</i>	PQ325655	PQ349968
FQD-13	Corn	<i>Fusarium verticillioides</i>	PQ325656	PQ349973
FQD-16	Corn	<i>Fusarium verticillioides</i>	PQ325657	PQ409349
FQD-17	Corn	<i>Fusarium verticillioides</i>	PQ325658	PQ362701
FQD-18	Corn	<i>Fusarium verticillioides</i>	PQ325659	PQ362700
FQD-20	Corn	<i>Fusarium verticillioides</i>	PQ325660	PQ409350
FQD-21	Corn	<i>Fusarium verticillioides</i>	PQ325661	PQ349969
FQD-22	Corn	<i>Fusarium verticillioides</i>	PQ325662	PQ349970
FQD-23	Corn	<i>Fusarium verticillioides</i>	PQ325663	PQ349972
FQD-24	Corn	<i>Fusarium verticillioides</i>	PQ325664	PQ369624
FOFMC-26	Wheat	<i>Fusarium</i> sp.	PQ325667	PQ330259
FOFMC-27	Wheat	<i>Fusarium</i> sp.	PQ325668	PQ330260
FQD-4	Corn	<i>Fusarium thapsinum</i>	PQ325666	PQ330258

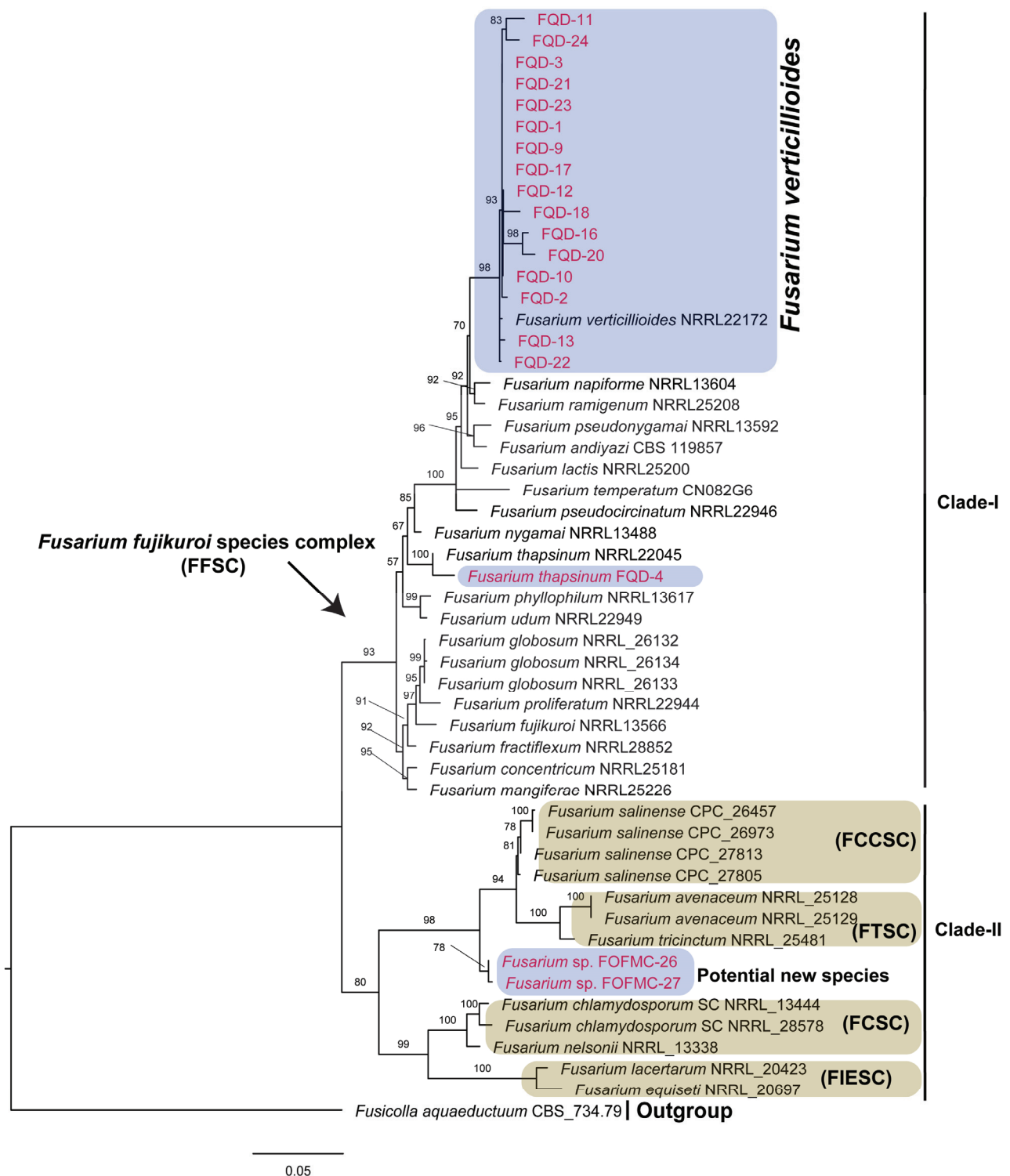


Figure 1. Maximum likelihood (ML) phylogeny of *Fusarium* species based on combined ITS-EF-1 α sequences, with *Fusicolla aquaeductuum* as the outgroup taxon. Species of the genus are recovered in two clades, where Clade-I consists of species *F. fujikuroi* species complex (FFSC), and Clade-II with *F. citricola* species complex (FCCSC), *F. tricinum* species complex (FTSC), *F. chlamydosporum* species complex (FCSC), and *F. incarnatum-equiseti* species complex (FIESC). There were 16 isolates of *F. verticillioides* recovered from corn (10 isolates), barley (4 isolates) and wheat (2 isolates), one isolate of *F. thapsinum* from corn, and two isolates of a potential new species of *Fusarium* from wheat. The bootstrap (BT) percentages above 50% are shown above nodes, all the newly generated sequences are in blue highlighted shade in red fonts.

3.6. Mycotoxin Production by *Fusarium* spp.

These *Fusarium* isolates produced varying levels of mycotoxins (Table 6). The *Fusarium* species produced FUM at levels ranging from 49.7 to 2489.2 $\mu\text{g kg}^{-1}$. *F. verticillioides* isolate FQD-1 produced the highest level of FUM (2489 $\mu\text{g kg}^{-1}$), followed by FQD-20 (2408 $\mu\text{g kg}^{-1}$), FQD-17 (1895 $\mu\text{g kg}^{-1}$), and FQD-13 (1044 $\mu\text{g kg}^{-1}$). No FUM production was detected in *F. verticillioides* isolates FQD-3, FQD-9, and FQD-10; *F. thapsinum* isolate FQD-4; and *Fusarium* sp. isolates FOFMC-26 and FOFMC-27. The production of ZEN by the *Fusarium* spp. ranged from 2.5 to 9.2 $\mu\text{g kg}^{-1}$. The maximum level of ZEN was produced by *F. verticillioides* FQD-20 (9.2 $\mu\text{g kg}^{-1}$), followed by *F. verticillioides* FQD-2 (2.8 $\mu\text{g kg}^{-1}$), and *Fusarium* sp. FOFMC-26 (2.5 $\mu\text{g kg}^{-1}$). All other isolates tested showed no ZEN production. All tested *Fusarium* strains produced DON, with levels ranging from 26 to 213 $\mu\text{g kg}^{-1}$. *F. thapsinum* FQD-4 produced the highest level (213 $\mu\text{g kg}^{-1}$) of DON, followed by *F. verticillioides* FQD-13 (192 $\mu\text{g kg}^{-1}$) and *Fusarium* sp. FOFMC-26 (188 $\mu\text{g kg}^{-1}$). The control samples (uninoculated corn substrate) showed no detectable levels of FUM, ZEN, and DON.

Table 6. Mycotoxin production by *Fusarium* species from cereal grains.

Fungal Isolates	Deoxynivalenol ($\mu\text{g kg}^{-1}$)	Fumonisin ($\mu\text{g kg}^{-1}$)	Zearalenone ($\mu\text{g kg}^{-1}$)
<i>Fusarium verticillioides</i> FQD-1	69.2 $\pm$ 4.5	2489.2 $\pm$ 27.3	nd
<i>Fusarium verticillioides</i> FQD-2	147.2 $\pm$ 13.2	336.5 $\pm$ 21.0	2.8 $\pm$ 0.1
<i>Fusarium verticillioides</i> FQD-3	108.2 $\pm$ 4.6	nd	nd
<i>Fusarium verticillioides</i> FQD-9	77.1 $\pm$ 8.6	nd	nd
<i>Fusarium verticillioides</i> FQD-10	161.0 $\pm$ 4.0	nd	nd
<i>Fusarium verticillioides</i> FQD-11	27.7 $\pm$ 0.9	379.6 $\pm$ 21.0	nd
<i>Fusarium verticillioides</i> FQD-12	54.1 $\pm$ 2.4	106.0 $\pm$ 1.7	nd
<i>Fusarium verticillioides</i> FQD-13	192.0 $\pm$ 9.9	1044.6 $\pm$ 45.1	nd
<i>Fusarium verticillioides</i> FQD-16	104.6 $\pm$ 12.7	173.9 $\pm$ 3.9	nd
<i>Fusarium verticillioides</i> FQD-17	67.7 $\pm$ 5.2	1895.5 $\pm$ 23.7	nd
<i>Fusarium verticillioides</i> FQD-18	133.3 $\pm$ 2.6	229.6 $\pm$ 60.9	nd
<i>Fusarium verticillioides</i> FQD-20	96.7 $\pm$ 11.8	2408.1 $\pm$ 32.9	9.2 $\pm$ 0.0
<i>Fusarium verticillioides</i> FQD-21	31.1 $\pm$ 0.7	137.8 $\pm$ 42.3	nd
<i>Fusarium verticillioides</i> FQD-22	25.6 $\pm$ 0.4	49.7 $\pm$ 5.1	nd
<i>Fusarium verticillioides</i> FQD-23	29.4 $\pm$ 0.9	101.4 $\pm$ 1.0	nd
<i>Fusarium verticillioides</i> FQD-24	149.0 $\pm$ 15.9	92.1 $\pm$ 13.2	nd
<i>Fusarium</i> sp. FOFMC-26	188.3 $\pm$ 23.6	nd	2.5 $\pm$ 0.1
<i>Fusarium</i> sp. FOFMC-27	68.5 $\pm$ 1.4	nd	nd
<i>Fusarium thapsinum</i> FQD-4	213.0 $\pm$ 9.8	nd	nd
Control	nd *	nd	nd

Data are means $\pm$ standard deviation from triplicate analysis. * Not detected, refers to values below quantification limit of assay.

Among the *Fusarium* isolates tested, *F. verticillioides* FQD-20 produced high levels of both FUM and ZEN. *F. thapsinum* FQD-4, which produced the highest level of DON, did not produce FUM and ZEN. Similarly, *F. verticillioides* FQD-1, which produced the highest level of FUM, produced no ZEN and low levels of DON.

4. Discussion

Food contamination by *Fusarium* toxins poses serious health and economic problems globally. For example, the natural occurrence of *Fusarium* toxins in wheat and corn varied significantly between China's high- and low-risk areas for esophageal cancer, according to Luo et al. [65]. Both the incidence and average levels of DON were higher in the high-risk areas compared to the low-risk areas. Hence, the occurrence of *Fusarium* toxins in agricultural commodities has been monitored worldwide [65–77]. In this study, out of 90 cereal grain samples consisting of wheat, corn, rice, and barley, only 3 samples (3.3%), one wheat and two corn, exceeded the EU's maximum permissible limit for ZEN

(200 $\mu\text{g kg}^{-1}$) and FUM (2000 $\mu\text{g kg}^{-1}$), respectively. DON levels remained within the permissible limits (1750 $\mu\text{g kg}^{-1}$). FUM, ZEN, and DON levels in 81%, 97%, and 44% of the samples, respectively, were below the detection limits.

Several studies reported the prevalence of *Fusarium* toxins in agricultural commodities [17,27,78,79]. Scudamore and Patel [71], while analyzing 82 consignments of maize imported into the UK for *Fusarium* toxins, reported that fumonisins were detected in almost every sample, and the maximum concentrations found for deoxynivalenol, nivalenol, zearalenone, and fumonisin B1 + fumonisin B2 were 444, 496, 165, and 5002 ppb, respectively. Logrieco et al. [66] reported that wheat and maize collected from Mediterranean countries were contaminated with DON, with levels reaching up to 8 mg kg^{-1} in wheat and 30 mg kg^{-1} in maize. Vrabcheva et al. [68] reported that DON and ZEA were the predominant toxins in wheat samples from Bulgaria, with contamination frequencies of 67% and 69%, respectively. The average levels in the contaminated samples were 180 $\mu\text{g kg}^{-1}$ for DON and 17 $\mu\text{g kg}^{-1}$ for ZEA, with maximum concentrations of 1800 $\mu\text{g kg}^{-1}$ and 120 $\mu\text{g kg}^{-1}$, respectively. In Korea, Lee et al. [80] examined polished rice, brown rice, and two kinds of by-products, viz., discolored rice and blue-tinged (less-ripe) rice, for *Fusarium* mycotoxins. DON (59 to 1355 ng g^{-1}), Nivalenol (NIV) (66 to 4180 ng g^{-1}), and ZEA (25 to 3305 ng g^{-1}) were found in rice samples that had discolored. ZEA (26 to 3156 ng g^{-1}), NIV (50 to 3607 ng g^{-1}), and DON (86 to 630 ng g^{-1}) were detected in blue-tinged rice. The main contaminants found in brown rice samples were ZEA (47 to 235 ng g^{-1}) and NIV (52 to 569 ng g^{-1}). However, samples of polished rice were mostly devoid of mycotoxins. Birr et al. [81] analyzed forage maize samples collected from Northern Germany and reported high incidences of DON and ZEN (100 and 96%, respectively), with concentrations reaching up to 10,972 $\mu\text{g kg}^{-1}$ for DON and 3910 $\mu\text{g kg}^{-1}$ for ZEN. Deoxynivalenol-3-glucoside (DON3G) and 3- and 15-acetyl-deoxynivalenol (3 + 15-AcDON), two modified forms of DON, were also found in almost all samples (100 and 97%, respectively), with maximal values of 3038 $\mu\text{g kg}^{-1}$ and 2237 $\mu\text{g kg}^{-1}$. Additionally, ZEN metabolites, α - and β -zearalenol (α -ZEL and β -ZEL), were found in lower incidences (59 and 32%), with concentrations up to 423 $\mu\text{g kg}^{-1}$ and 203 $\mu\text{g kg}^{-1}$, respectively. The natural presence of *Fusarium* mycotoxins in barley and corn samples from Korea was examined by Kim et al. [82]. They discovered that the main contaminants in barley were DON, NIV, and ZEA, with mean concentrations of 170 ng g^{-1} , 1011 ng g^{-1} , and 287 ng g^{-1} , respectively. With mean concentrations of 310 ng g^{-1} and 297 ng g^{-1} , respectively, DON and 15-acetyldeoxynivalenol (15-ADON) were the main contaminants in corn. Shantika et al. [77] reported that more than 35% of maize food products collected from Indonesia markets contained both DON and FUM simultaneously. DON was found in 42.2% of the samples, but FUM was found in 84.4%. FUM and DON mean values varied from 0.12 $\mu\text{g kg}^{-1}$ to 264.24 $\mu\text{g kg}^{-1}$ and 2.62 $\mu\text{g kg}^{-1}$ to 122.28 $\mu\text{g kg}^{-1}$, respectively. In their analysis of wheat grain samples gathered from Nakuru and Nyandarua, Kenya, Muthomi et al. [70] discovered that the majority of the samples were tainted with mycotoxins, with occurrence rates of up to 86% for T-2 toxin and 75% for deoxynivalenol (DON). Zearalenone and aflatoxin B1 were among the other mycotoxins found. Up to 35% of the samples had DON, T-2 toxin, and zearalenone co-occurring. Chilaka et al. [75] investigated maize, sorghum, millet, and *ogi* (akamu) samples from Nigerian markets for *Fusarium* mycotoxins contamination. Their findings revealed that fumonisins were the most common, particularly in maize and *ogi*, with occurrence rates of 65% and 93% and mean concentrations of 935 and 1128 $\mu\text{g kg}^{-1}$, respectively. In addition, multiple toxins were found to be present in 43% of the samples. Stanciu et al. [76] investigated the presence of *Fusarium* mycotoxins in Romanian wheat flour and grains. They reported that DON was detected in 14% of the samples, containing levels between 111 and 1787 $\mu\text{g kg}^{-1}$, while ZEN was present in 9% of the samples, with levels between 51 and 1135 $\mu\text{g kg}^{-1}$. The low levels of mycotoxin contamination in the cereal grain samples in this study might be due to strict import regulations on food commodities and monitoring and good storage practices followed by the traders.

In this study, nineteen *Fusarium* isolates were found in the tainted grain samples and identified by PCR. On the basis of combined analysis of ITS and EF-1 α sequences, these fungal isolates were identified as *F. verticillioides* (16 isolates), *F. thapsinum* (1 isolate), and new *Fusarium* species (2 isolates). Chrpova et al. [83] detected the presence of *F. poae* and *F. graminearum* in winter wheat from the Czech Republic. They found that DON production by *F. graminearum* and *F. culmorum* was significantly elevated in instances of mixed infections with other species. *F. graminearum* was the most often observed species in wheat grown in the Netherlands, according to Van der Fels-Klerx et al. [72], followed by *F. avenaceum* and *Microdochium nivale*. The incidence and concentrations of DON were the highest, followed by ZEN and beauvericin. According to Lee et al. [80], the blue-tinged rice in Korea had the highest contamination rate with *F. graminearum* (3.8%), followed by discolored rice (2.4%), and brown rice (1.6%). According to Muthomi et al. [70], samples of freshly harvested wheat grain from Kenya's Nakuru and Nyandarua exhibited high levels of fungal contamination, particularly from *Epicoccum*, *Alternaria*, and *Fusarium* species. Isolates of *F. graminearum* demonstrated high virulence and significantly reducing kernel weight.

Numerous mycotoxins, such as FUM, ZEN, fusaric acid, T-2 toxin, HT-2 toxin, and DON, are known to be produced by *Fusarium* species [66,84–88]. Shi et al. [64] reported that *F. proliferatum*, *F. verticillioides*, *F. solani*, and *F. fujikuroi* produced fumonisins and fusaric acid; *F. oxysporum*, *F. temperatum*, *F. subglutinans*, *F. tricinctum*, *F. equiseti*, *F. concentricum*, *F. musae*, *F. andiyazi*, and *F. sacchari* produced only fusaric acid; *F. sporotrichioides*, *F. polyphialidicum*, and *F. langsethiae* produced type A trichothecenes; and *F. graminearum*, *F. poae*, *F. culmorum*, and *F. meridionale* produced type B trichothecenes. The *Fusarium* species isolated in this study produced FUM at levels ranging from 49.7 to 2489.2 $\mu\text{g kg}^{-1}$. *F. verticillioides* isolates exhibited the highest production of FUM. The production of ZEN by the *Fusarium* spp. ranged from 2.5 to 9.2 $\mu\text{g kg}^{-1}$. Among them, *F. verticillioides* FQD-20 yielded the highest concentration at 9.2 $\mu\text{g kg}^{-1}$. All *Fusarium* spp. tested produced DON, with concentrations ranging from 26 to 213 $\mu\text{g kg}^{-1}$. *F. thapsinum* FQD-4 recorded the highest DON level at 213 $\mu\text{g kg}^{-1}$, followed by *F. verticillioides* FQD-13 at 192 $\mu\text{g kg}^{-1}$ and *Fusarium* sp. FOFMC-26 at 188 $\mu\text{g kg}^{-1}$. DON is primarily produced by *F. graminearum* and *F. culmorum* [89,90]. When examining the toxigenic potential of certain *Fusarium* isolates from Mediterranean cereals, Logrieco et al. [66] found that *F. culmorum* produced ZON, α - and β -ZOH, DON, and 3-AcDON; *F. graminearum* produced ZON, DON, and 15-AcDON; *F. crookwellense* produced ZON, α - and β -ZOH, FUS, and NIV; *F. equiseti* produced ZON and α -ZOH; and *F. avenaceum* produced moniliformin. None of the isolates of *F. moniliforme*, *F. proliferatum*, and *F. semitectum* were able to produce mycotoxins. However, Ramakrishna et al. [91] reported that *F. moniliforme* (syn: *F. verticillioides*) produced DON under optimum conditions, albeit at low concentrations (0.01–0.09 $\mu\text{g g}^{-1}$ liquid medium). In this study, *F. thapsinum* FQD-4 isolated from corn produced the highest level of DON. *F. thapsinum* is a pathogen that causes stalk rot and grain mold of sorghum [92] and stalk rot of maize [93]. It is known to produce high levels of moniliformin and fusaric acid [94]. Leslie et al. [95] reported that *F. thapsinum* recovered from sorghum produced little to no fumonisins but substantial amounts of moniliformin. Considering the economic significance of corn in Oman and globally, *F. thapsinum* may be emerging as an important mycotoxigenic fungus.

5. Conclusions

This study revealed that *Fusarium* toxin levels in 97% of the cereal grain samples collected from Muscat, Oman, were within the permissible limits set by the European Commission. However, 13% of corn samples and 3% of wheat samples exceeded the maximum permissible limit for FUM and ZEN, respectively. The co-occurrence of these mycotoxins could pose health risks to local consumers due to their potential synergistic or additive effects. *Fusarium* species are a significant source of mycotoxins in food and feed. Furthermore, the presence of toxigenic *Fusarium* strains in certain grain samples could serve as a source of inoculum, potentially leading to disease outbreaks in wheat crops across various wilayats (provinces) in Oman, where wheat is cultivated. The mold growth and

mycotoxin contamination could pose a threat to the quality of cereal grains. Oman imports most cereal grains from other countries, and hence, stricter quarantine regulations are essential to mitigate the risk of mycotoxin contamination. Enhancing consumer awareness about mycotoxins, ensuring proper storage conditions, and promoting food safety practices are essential for preventing *Fusarium* growth and toxin production in cereal grains. Further investigation is needed to ascertain the production of other mycotoxins by these *Fusarium* species. As far as we are aware, this is the first report documenting the presence of *Fusarium* spp. and their associated mycotoxins in cereal grains sold in Oman.

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Review

Progress in Research on Prevention and Control of Crop Fungal Diseases in the Context of Climate Change

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Abstract: With an advancement in global climate change, the frequency of extreme climatic events, such as high temperature, drought, and flooding, has increased. Meanwhile, outbreaks of crop fungal diseases are becoming more frequent and serious, and crop growth and food production are seriously threatened. This article focuses on the climate change-related aggravation of crop fungal diseases; summarizes the progress in research on the impact of climate change on soil-borne fungal diseases, air-borne fungal diseases, and seed-borne fungal diseases; and discusses the conventional methods for diagnosing crop fungal diseases. On the basis of comparative analysis, the concept of ecological control is proposed; ecological control can maintain the dynamic balance of crop–biology–soil, provide a good environment for the healthy growth of crops, and provide a new fungal disease control method in the context of climate change.

Keywords: climate change; crops; fungal diseases; advances

1. Introduction

By 2050, the world's population will exceed 9.7 billion, and human demand for food will continue to grow. However, crop yields globally have suffered severe declines due to disease, with 70–80% of crop diseases being caused by fungi [1]. There are approximately 1.5 million fungal species globally, of which *Fusarium* alone causes wilt and root rot in more than 100 crops [2], such as tomato wilt [3], corn rusts [4], and wheat fusarium head blight [5]. Indeed, most crops are susceptible to fungal diseases, with rice blast [6], wheat common smut, and wheat stem rust [7] causing 10–35% and 15–20% yield losses in rice [8] and wheat [9]. Although fungal diseases already pose a serious threat to global food security [10], the advancement of climate change has deepened this threat, highlighting the urgency of the challenge that this poses for humans.

It is widely recognized that climate change has the potential to decrease the productivity of food crops [11–14]. With global average temperatures having risen by 1.5 °C since the industrial revolution. The model predicts that an increase in temperature of 1.6 °C–3 °C will result in an 18–32% reduction in potato yields [15], which is particularly concerning given that the median global temperature is projected to rise by 2.6 °C–3.1 °C in 2100 [16,17]. As such, the reductions in crop yields seen to date may become far more severe in the future. In addition, climate change stimulates the growth of fungi that are pathogenic to crops and increases the incidence of fungal diseases. Higher temperatures

would increase the relative abundance of most fungal pathogens globally [1], and fungal pathogens are more aggressive at higher temperatures (Figure 1) [18]. Flooding also promotes the spread of pathogenic fungi through soil runoff. For example, it has been shown that *Colletotrichum* rapidly infects and causes disease after reaching onion cultivations through rainfall washout transmission [19]. In addition, an increase in relative humidity due to flooding can trigger outbreaks of leaf spot or other fungal diseases in winter wheat [20]. It has also been reported that under drought and salt stress conditions, the rate of infection of broad bean root rot increases to 25–100% [21].

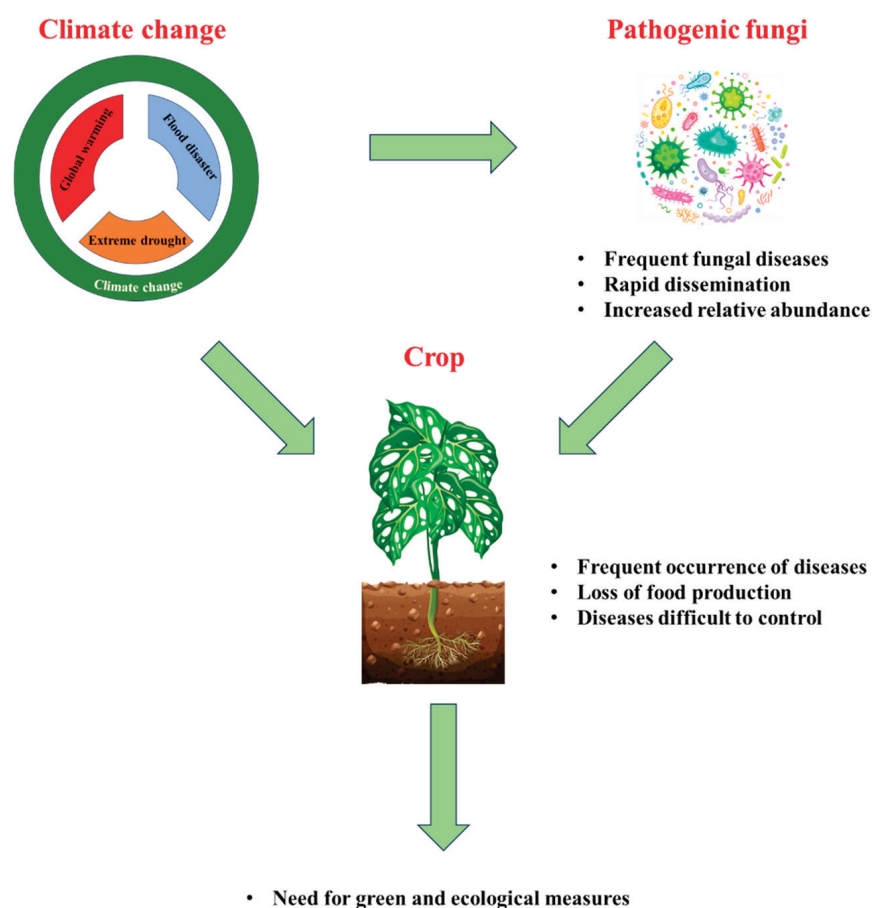


Figure 1. Relationship between climate, crops, and fungal pathogens.

Overall, in the context of global climate change, fungal diseases of crops become established more rapidly, spread over a wider area, and are more damaging [18]. Moreover, the chemical pesticides that are currently most commonly used are no longer adequate because they damage plants and lead to resistance of pathogenic fungi and environmental destruction [22,23]. This background has prompted researchers to rethink microbe–crop–soil interactions as a whole to achieve ecological prevention and control of fungal diseases. This article focuses on the climate change-related aggravation of crop fungal diseases; summarizes the progress of research on the impact of climate change on soil-borne, air-borne, and seed-borne fungal diseases; discusses the methods for diagnosing crop fungal diseases; and proposes ecological prevention and control methods for maintaining crop health and preventing and controlling crop fungal diseases based on comparative analyses of such approaches.

2. Climate Change Exacerbates Crop Fungal Diseases

Increased frequency of extreme weather events due to climate change, such as droughts, floods, and high temperatures, has exacerbated the prevalence of crop fungal diseases at an alarming rate. This has in turn affected the growth cycle, yield, and quality of crops [24,25].

2.1. Drought Exacerbates Crop Fungal Diseases

Drought stress reduces crop fine roots and soil microbial diversity, allowing disease-causing fungi to infect crops more readily [26]. Studies have shown that drought exacerbates faba bean wilt [21], chickpea root rot [27], rice blast [28], and grapevine disease [29]. The lack of attention and efforts to prevent and control soil-borne pathogens in arid areas makes them more likely to cause significant damage [30]. It was reported that an annual drought in May–June significantly increases the incidence of rust in winter wheat in Poland [31]. Moreover, drought stress-induced soil salinization has been reported to increase the susceptibility of crops to pathogens and promote the spread of fungal crop diseases [32]. The interaction between crop salt stress and pathogen infection depends on the type of pathogen and the host crop species. For example, onions exposed to salt stress conditions suffer from more severe infestation with *Fusarium oxysporum* than those not exposed to salt stress [33,34]. Moreover, increased salinity of irrigation water significantly exacerbated cucumber damping-off disease caused by *Pythium aphanidermatum* [35].

2.2. Flooding and High Humidity Exacerbates Crop Fungal Diseases

Extreme precipitation can lead to frequent flooding and an increase in pathogenic fungi [31]. For example, precipitation or soil runoff can promote the spread of disease via spores of pathogenic fungi in the soil and in the air [36]. Prolonged rainfall makes it easier for *Fusarium* to attack wheat, which is more susceptible to tan spot disease [37]. Indeed, dramatic increases in the levels of pathogens such as *Phytophthora*, *Fusarium*, and *Pythium* have been reported after flooding [38]. In addition, the transport and agglomeration of fungal spores of *Epicoccum tritici* were found to be promoted by water condensation and evaporation [39]. Moreover, sugarcane is known to be more susceptible to false floral smut under conditions of high precipitation or high humidity [40], while the incidence of cashew leaf blight was shown to increase by 2% for every 1 mm increase in precipitation [41]. The number of spots in chickpea was also found to increase with increasing rainfall [42], while the incidence and severity of quinoa downy mildew increased under flooded conditions [43]. Finally, it was found that the likelihood of rice leaf blight and brown spot occurring simultaneously increases with increasing soil water content [44].

2.3. High Temperatures Exacerbate Crop Fungal Diseases

High temperatures are among the most direct manifestations of climate change, and prolonged high temperatures can increase the extent and severity of crop diseases [45]. For example, elevated temperatures can lead to pathogen spore germination, crop root damage, and an increased incidence of disease in susceptible crops. For example, the germination time of *Botryosphaeria* as the causative agent of grapevine trunk disease was found to be shorter at elevated temperatures [46], exacerbating this disease [47]. High temperatures were also shown to increase the incidence of root rot in celery, mainly due to the reduced expression of *Fusarium* resistance [48]. Soybean was also reported to suffer a higher incidence of *Fusarium solani*-induced root diseases at 30 °C [49], and soybean is more susceptible to sudden death syndrome at 37 °C, indicating that disease transmission is sensitive to temperature. Temperature changes also cause spot growth, increased infection efficiency, and spore formation of potato late blight, along with increases in infection efficiency, spot growth rate, and spore formation occurring under fluctuating temperature conditions compared with the rates at a constant temperature [50]. Moreover, elevated storage temperature also exacerbates crop fungal diseases, with a significant growth in the rice mold population being identified after 16 weeks of storage at 40 °C [51], showing incidence rates as high as 96.5%–99.1%. Moreover, while no decay occurred in carrots at a storage temperature of 10 °C, decay was greatest when the temperature rose to 35 °C [52]. It has also been found that increasing storage temperature from 2 °C to 25 °C increased the risk of infection of nectarines with brown rot by more than 60-fold [53] (Table 1).

Table 1. Prevalence of crop fungal diseases due to climate change.

Type of Climate Change	Crop	Disease	Pathogenic Fungus	Hazard	Reference
Desertification	Faba bean	Root rot	<i>Fusarium equiseti</i> , <i>Fusarium graminearum</i> , <i>Fusarium brachygibbosum</i>	Growth rate of pathogenic fungi increased by 30–44%	[21]
	Chickpea	Root rot	<i>Macrophomina phaseolina</i>	Chickpea production down 66.99%	[27]
	Rice	Rice blast	<i>Magnaporthe oryzae</i>	Plants under drought for longer have higher fungal abundance and poorer germination	[28]
Flooding and high humidity	Cucumber	Damping-off disease	<i>Pythium aphanidermatum</i>	Significant increase in morbidity from 40% to 93%	[35]
	Sugar cane	False floral smut	<i>Epicoccum andropogonis</i> , <i>Claviceps purpurea</i>	Heavy precipitation during the bloom period resulted in a higher incidence than in previous years	[40]
	Cashew	Leaf blight	<i>Cryptosporiopsis</i> spp.	2% increase in incidence per mm increase in rainfall	[41]
	Quinoa	Downy mildew	<i>Peronospora variabilis</i>	Decreased chlorophyll in quinoa after disease onset	[43]
High temperatures	Grapevine trunk	Grapevine trunk disease	<i>Lasiodiplodia theobromae</i>	Pathogenic fungi grow at 4 °C–40 °C and prefer higher temperatures	[46]
	Celery	Root rot	<i>Fusarium oxysporum</i>	<i>Fusarium acanthamoeba</i> levels and disease severity increased with increasing temperature	[48]
	Soybean	Root rot	<i>Fusarium solani</i>	Longer root lesion lengths and higher disease incidence of <i>Fusarium</i> at 30 °C	[49]

3. Impact of Climate Change on Crop Fungal Diseases with Different Routes of Transmission

Crop fungal diseases are mainly categorized into soil-borne fungal diseases, air-borne fungal diseases, and seed-borne fungal diseases according to the transmission route. Climate change can alter the sites of infestation, disease levels, and mechanisms by which fungi cause damage via different transmission routes.

3.1. Soil-Borne Fungal Diseases

Soil-borne fungal diseases occur when fungi residing in the soil alongside crop remnants invade crop roots or stems under favorable conditions. These diseases hinder root functionality and the absorption of water and nutrients, resulting in stunted growth and decreased crop yields [54]. Especially in cases where there are continuous cropping and soil degradation, outbreaks of soil-borne diseases can frequently occur [55]. For example, potato late blight causes annual losses of approximately EUR 6.1 billion globally [56], and potatoes are more susceptible to extreme summer temperatures and drought [57]. The ratio of mild evapotranspiration to precipitation limits crop diversity, and extreme summer temperatures and drought make it easier for pathogens to attack crops, with some soil-borne pathogens, such as *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., and *Mycosphaerella* spp., leading to crop yield reductions of 50–75% [58]. Climate change causes soil acidification,

which alters the physicochemical properties and microbial communities of soil, promoting the accumulation of pathogens and exacerbating soil-borne diseases [59]. Acidic soils are in turn associated with the greatest incidence of *Fusarium*-induced root rot diseases of soybeans. Extreme climate conditions predispose crops to injury, and such vulnerable crops are more susceptible to disease. It has been reported that temperatures above 30 °C trigger oxidative stress in grains, which directly damages enzymes and tissues [60], and fungi such as *Aspergillus* and *Fusarium oxysporum* often parasitize and proliferate through sites of damage in crops [61]. There is increasing evidence to suggest that soil-borne diseases are not solely caused by individual species, but rather by pathogenic complexes [62–65], and the complexity of soil-borne pathogen–environment interactions is highlighted by the existence of multifaceted and contrasting interactions, such as those with temperature and humidity [66]. This complexity is further aggravated by the unpredictable and fluctuating proportions of diverse pathogens, which vary significantly across seasons and regions [67].

3.2. Air-Borne Fungal Diseases

Air-borne fungal diseases involve the spread of crop pathogens via spores aided by air currents to cause crop disease epidemics and associated damage. Pathogenic fungi, such as *Cladosporium*, *Penicillium*, and *Aspergillus* (including the species *Aspergillus fumigatus*), are present in the air [68] and cause air-borne fungal diseases including gray mold, downy mildew, powdery mildew, and early blight [69]. Individuals or groups of pathogens that are rapidly transmitted through the air can cause a wide range of diseases, intense outbreaks, and serious disasters. The spread of yellow rust to Australia in 1978 is an example of the spread of an air-borne disease [70], where the original rust fungus originated in Europe [71,72]. Another case of this kind involved Australian wheat stem rust, which occurred for the first time in Ethiopia in the mid-1980s, followed by its gradual emergence in the Middle East and the Indian subcontinent [73,74]. Increased air-borne sporulation is driven by higher temperatures and increased humidity. Studies have shown that temperature is the most important variable for the spread of *Alternaria* spores in Spain [75]. Meanwhile, it has been reported that warm climatic conditions in France promote the rapid growth of *Cladosporium* fungal spores [76]. Spores are transported over long distances by diffusion into the surrounding area through droplets or air [77]. Cucurbit downy mildew caused by the oomycete Cuban pseudofungus spreads rapidly [78], and it has been shown that relative humidity above 90% and a temperature between 15 °C and 20 °C are the optimal conditions for downy mildew [79], leading to yield losses of up to 80% in European cucurbits [78,80]. Another example of an air-borne fungal disease is black spot caused by *Venturia inaequalis*, which is an important disease of apples worldwide [81] and can cause 70% yield loss [81–83]. Meanwhile, early blight caused by *Alternaria solani* is a common disease of potato [84,85] that causes great damage under high-temperature conditions [86,87]. It has been reported that early blight is likely to become more severe in European potato-cultivating areas in the future [88].

3.3. Seed-Borne Fungal Diseases

Seed-borne fungal diseases are those in which the seed produced by a crop carries a pathogenic fungal propagule that develops at a certain stage of growth after the seed has been sown when suitable climatic conditions prevail. The fungal pathogens can be present on the outside or inside of the seed and may cause seed septoria, rot, and necrosis as well as impairing seedling growth [89]. When a crop pathogenic fungus binds to the seed, it may cause significant economic losses [90]. For example, when healthy wheat seeds are infested with *Tilletia caries*, even when sown into uninfested soil, the seed germinates and the latent pathogen invades the wheat spike as it spreads through the wheat seed [56], resulting in reduced seed quality and toxic metabolite production [91]. *Aspergillus flavus*, the seed-borne fungal pathogen of mungbean, is a toxic pathogenic fungus whose pathogenicity is primarily related to aflatoxin. It has been found that the rate of *A. flavus* colonization and the aflatoxin concentration in peanuts increase at temperatures close to 35 °C [92]. Increased

storage temperature and humidity can lead to seed ulceration. High relative humidity during storage conditions can lead to pathogenic fungal growth, which can negatively impact the germination and growth of rice seeds [93]. Meanwhile, with increasing water content or storage temperature of soybean seeds, pathogenic fungi produce chemicals that accelerate seed deterioration [94] (Table 2).

Table 2. Mechanisms by which climate change impacts fungal diseases with different transmission routes.

Transmission Route	Crop	Pathogenic Fungus	Mechanisms of Influence	Reference
Soil-borne disease	Tomato	<i>Fusarium solani</i> , <i>F. oxysporum</i> or <i>Ilyonectria destructans</i>	Crops are more susceptible to extreme summer temperatures and drought	[54,57–59]
	Maize	<i>Fusarium graminearum</i>	High temperatures trigger oxidative stress in grains that damages enzymes and tissues, making fungi more susceptible to infestation	[61]
Air-borne disease	Cucurbit	<i>Pseudoperonospora cubensis</i>	Relative humidity > 90% and temperature 15 °C–20 °C are optimal conditions for cucurbit downy mildew development	[78,79]
	Potato	<i>Alternaria solani</i>	<i>Streptomyces</i> levels exceed 50 spores/m ³ at average temperature > 18 °C and leaf humidity > 80%	[84,85]
Seed-borne disease	Wheat	<i>Tilletia caries</i>	Stored seeds are affected by temperature and humidity	[91]
	Peanut	<i>Aspergillus flavus</i>	Increase in the proportion of <i>Aspergillus flavus</i> colonizing peanuts and aflatoxin concentration with increasing temperature	[92]
	Rice	<i>Fusarium fujikuroi</i>	At >25 °C, mycelium grows most vigorously	[93]

4. Control of Fungal Diseases of Crops

The precise diagnosis of fungal diseases is a prerequisite for improving the effectiveness of prevention and control of such diseases in crops. Different crop diseases, different transmission routes, and diseases at different reproductive periods require the adoption of highly sensitive detection technologies and means. Disease prevention and control are currently divided into disease-resistant breeding, agronomic measures, chemical control, biological control, and ecological control.

4.1. Disease Detection

4.1.1. Methods for Diagnosing Fungal Diseases of Crops

The timely detection of crop diseases and their reliable diagnosis can make a major contribution to maintaining total food production globally [95]. Methods for diagnosing fungal diseases in crops can be divided into two categories: traditional and modern detection methods. Traditional detection methods mainly rely on morphological, microbiological, and biochemical identification, with fungal pathogens being detected by visual inspection methods and culture methods. The results of visual inspections are greatly influenced by the knowledge and experience of the inspectors, and have limited accuracy in the on-site diagnosis of fungal pathogens of crops [96]. Meanwhile, the culture method is time-consuming, with 3–5 days usually being required to cultivate the fungus [97]. In conclusion, traditional detection methods are time-consuming, ineffective, and unsuitable when efficient and rapid detection is required. Meanwhile, modern detection methods can be subdivided into direct and indirect detection methods, which are the main current methods for diagnosing fungal diseases.

Direct Detection Methods

Direct detection methods can be categorized into immunological methods and polymerase chain reaction (PCR) methods. The principle of immunological methods is based on the binding of antigen and antibody, and these methods include the immunodiffusion test, enzyme-linked immunosorbent assay, radioimmunosorbent assay (RISA), and dot immunobinding assay (DIBA) [98]. In terms of their advantages over traditional culture methods, immunological methods have a short detection time, but they have the disadvantages of low detection sensitivity and accuracy, as well as interference by contaminants. Meanwhile, PCR methods can detect specific target nucleic acids of the target pathogens and improve the specificity, sensitivity, and speed of detection [99]. However, they have the disadvantages of a lack of standardization, the need for additional preparation of samples, the difficulty of on-site operation, the need for special equipment, and the high cost of detection [100]. Overall, in the context of climate change, the above two methods are not suitable for diagnosing fungal pathogens in crops.

Indirect Detection Methods

Indirect detection methods identify crop diseases not by directly identifying the pathogen but by detecting the physiological response of the crop to the pathogen. The main indirect detection techniques include spectroscopy, biosensor detection, gas chromatography and mass spectrometry detection, and imaging techniques.

- (1) **Spectroscopy.** Spectroscopy is used to assess the health of a crop by irradiating light of a specific wavelength onto the crop tissue and measuring the intensity of the light wavelengths reflected back. Examples of types of spectroscopy include fluorescence spectroscopy, visible spectroscopy, infrared spectroscopy, and nuclear magnetic resonance spectroscopy. Near-infrared spectroscopy can detect maize spot disease and olive leaf spot [101,102]. However, spectroscopic methods cannot detect diseases before they develop in crops and cannot detect multiple diseases occurring at the same time.
- (2) **Biosensors.** A sensor is an analytical device that converts chemical, physical, or biological information into a useful analytical signal. A biosensor is “an integrated receptor–transducer device capable of providing selective quantitative or semiquantitative analytical information using biometric elements” [103]. In recent years, biosensors have attracted much attention due to their good results in detecting, classifying, diagnosing, and quantifying crop diseases. Biosensors can be categorized into optical biosensors, volatile biosensors, electrochemical biosensors, and mass-sensitive biosensors. Despite the ability of biosensors to rapidly and accurately detect disease-causing fungal pathogens, only a few devices are commercially available for detecting crop fungal pathogens in crops [104].
- (3) **Gas chromatography coupled with mass spectrometry (GC–MS).** GC–MS is a technique for analyzing volatiles produced by fungal infections in crops [105]. It has the advantages of not destroying the crop and enabling continuous monitoring for a long time [106], but it has the disadvantages of environmental factors such as humidity potentially interfering with the sensor readings [107] and the need to collect volatile organic compound samples prior to the GC–MS analysis, which limits its application in the field.
- (4) **Imaging technology.** Imaging technologies include thermal, hyperspectral, and red–green–blue (RGB) and fluorescence imaging, coupled with unmanned aerial vehicles (UAVs) that can monitor large farms. Using such technologies, diseases are diagnosed indirectly by detecting changes in the color, texture, and temperature of crop leaves. In this context, intelligent agricultural machines and robots can detect crop diseases at an early stage and monitor disease development remotely [108,109]. Meanwhile, aerial remote sensing (RS) using UAVs or unmanned aerial systems with intelligent vision systems is an efficient and inexpensive way of detecting diseases in crops [108–113]. It is reported that the integration of RGB, multispectral, hyperspectral, fluorescence, and

thermal infrared imaging sensors, coupled with efficient algorithms on unmanned aerial vehicles (UAVs), enables effective detection, differentiation, and quantification of the severity of symptoms induced by diverse pathogens under field conditions [114,115]. It has been shown that these methods can achieve reliable diagnosis in cereal crops, such as rice [116], corn [117], and wheat [118,119]; vegetables, such as grapevines [120], potatoes [121], soybeans [122], and tomatoes [123]; and forests, such as pine forests [108]. The combination of imaging obtained by drones with artificial intelligence algorithms, such as machine learning, is a promising new approach that not only enables early and accurate diagnosis of crop diseases but also improves crop yields while reducing the cost of disease treatment [124]. Utilizing RS technology to retrieve and gather data, encompassing meteorological information as well as the properties of the earth's surface and soil, and offering timely feedback, holds significant promise as an effective approach for diagnosing fungal crop diseases in the context of climate change [125] (Table 3).

Table 3. Methods for diagnosing crop fungal diseases.

Method Category	Diagnostic Method	Principle	Advantages and Disadvantages	Reference	
Traditional detection methods	Visual inspection method	Isolation of pathogens and interpretation of visual symptoms of disease through microscopy	Highly subjective and error-prone	[96]	
	Culture	Morphological characterization of pathogens by medium culture and microscopic observation	Cheap, simple, but accuracy and reliability depend on experience and skill, time-consuming	[97]	
Modern testing methods	Direct detection methods	Immunological methods	Based on antigen–antibody binding	Short detection time, low sensitivity and accuracy	[98]
		Polymerase chain reaction (PCR)	Detection of target nucleic acids specific to the target pathogen	Fast detection, lack of standardization, additional sample preparation, difficult on-site operation, high detection cost	[99,100]
	Indirect detection methods	spectroscopy	Measurement of the intensity of reflected light wavelengths irradiated by a specific light source to assess the health of the crop	Inability to detect disease prior to onset, and inability to detect multiple diseases occurring at the same time	[101,102]
		Biosensor detection	Provides selective quantitative or semiquantitative analytical information using biometric elements	Quantitative information on crop diseases is available, but commercially available equipment is limited	[103,104]
		GC–MS	Assessment of changes in volatiles due to fungal infection of crops	No crop damage, continuous monitoring over long periods of time, environmental factors interfere with sensor readings, pre-collection of samples required	[105–107]
		Imaging technology	Indirect diagnosis of diseases by detecting changes in color, texture, or temperature of crop leaves	Efficient and inexpensive, suitable for climate change conditions	[108,109]

4.2. Prevention and Control of Crop Fungal Diseases

4.2.1. Breeding Measures for Disease Resistance

Disease resistance breeding is a method of breeding new crop varieties that develop strong resistance to diseases through targeted selection or alteration of certain genotypes. It is one of the most efficient and economical measures to cope with crop diseases. However, improving the disease resistance of crops can simultaneously affect their yield [126]. It

has been shown that disease resistance breeding enhances *Phytophthora* blight resistance while simultaneously reducing *Rhizobia* colonization, resulting in reduced crop yields [127]. High-quality resistance results in the targeted selection of pathogens, while the speed and scale of pathogen spread increase under extreme climatic conditions. Moreover, research devoted to particular resistant varieties is often overtaken by the emergence of pathogens with greater pathogenicity [128].

4.2.2. Agronomic Measures

Agronomic measures involve the creation of environmental conditions that promote crop growth and the survival and reproduction of beneficial bacteria, but that inhibit the occurrence of pathogenic fungi. Such measures can involve practices such as fertilization, irrigation, and tillage. The application of fertilizer to the soil can change its physical and chemical properties and create an environment suitable for the growth of beneficial microorganisms. The application of chitin amendments to the soil was reported to contribute to soil carbon and nitrogen cycling [129]. Meanwhile, the application of cow dung after ammonia fumigation was found to reduce the abundance of *Fusarium spinosum* in soil and its infestation in bananas by approximately 55% [130]. Although irrigation can greatly reduce the negative effects of drought [131], there is a lack of effective agronomic measures to cope with disease outbreaks due to climatic extremes of rainfall and flooding; agronomic measures such as crop rotation and intercropping do not have effects that are rapid enough to control fungal disease in the current season [132], and although deep tillage may promote the onset of, for example, leaf spot disease in tea trees [133], the process of applying fertilizers is labor-intensive and time-consuming [134] and fertilizers pose the risk of nutrient leaching [135]. The greatest pressure on irrigation is the severe shortage of freshwater resources available for this purpose [136].

4.2.3. Chemical Control

Chemical control, which involves killing pathogenic bacteria using chemical pesticides to avoid crop damage, is a common method of managing fungal disease and is used for both disease prevention and treatment of diseased crops [137–139]. Chemicals play an important role in increasing crop yield and quality, improving food safety, reducing microbial toxins, and extending the shelf life of crops [140,141]. However, chemicals can have harmful effects on plant and animal health and nature [142–144]. The application of pesticides poisons organisms in the soil and water bodies and reduces soil biodiversity [145]. A previous study showed that higher spraying of fungicide reduces the diversity of *Xylaria* spp. isolates, which are beneficial to crops [146]. It has also been shown that penthiopyrad induces oxidative damage and lipid metabolism, causes mitochondrial dysfunction, and leads to apoptosis in fish [147]. The intensive use of chemicals has also been found to lead to increased pathogen resistance [148], and long-term pesticide application has significantly reduced the effectiveness of control of *Zymoseptoria tritici*, *Colletotrichum falcatum*, and *Venturia inaequalis* [149,150]. Moreover, with climate extremes, such as flooding, more than 90% of pesticides are lost in the field, resulting in serious risks to ecosystems. Some pesticides may even exert effects lasting for decades [151,152].

4.2.4. Biological Control

Biological control, which entails the utilization of organisms like crops, insects, and microorganisms, serves as a natural means to mitigate a diverse range of diseases triggered by pathogens [153], is considered a safe and environmentally friendly strategy that can reduce or eliminate the utilization of chemical pesticides [154]. The main forms of biological control involve the application of microbial or bio-organic fertilizers and bio-antagonistic fungicides to the soil. In terms of the microorganisms used for biological control, the main ones include bacteria such as *Pseudomonas* spp. and *Bacillus* spp. [155], fungi such as *Penicillium* spp. and *Trichoderma* spp., and actinomycetes such as *Streptomyces* spp. Regarding the mechanisms by which biocontrol bacteria exert their effects, these include

the formation of biofilm by *Bacillus* spp. to promote crop growth, improvement of the microbial community in the soil, and inhibition of the growth of pathogenic bacteria [156]. In terms of how biocontrol fungi work, they can induce crop resistance, produce antimicrobial substances against pathogenic fungi (e.g., iron carriers, volatile organic compounds, solubilizing enzymes), and even occupy ecological niches in advance. For example, it was reported that *Chromolaena odorata* not only increases the height and shoot/root dry weights of melon plants to promote crop development, but also inhibits the wilt pathogen *Fusarium oxysporum* by 90% [157]. However, biological control has the shortcoming that the microorganisms used are susceptible to environmental influences, especially in extreme climatic conditions such as drought and high temperatures, leading to unstable prevention and control effects during the application of microbial agents.

4.2.5. Ecological Regulation

In the context of global climate change and the increasing frequency of extreme weather events, traditional singular control measures have become inadequate in effectively preventing and controlling fungal diseases in crops. At its core, ecological control involves a systematic approach to provide a favorable environment for healthy crop growth and maintain the dynamic balance among crop–biology–soil (Figure 2). Soil health is linked to crop health. Heavy metal-stressed vetiver fractionates heavy metals in the soil by secreting organic acids, while raising the level of organic carbon and the pH of soil [158]. Crops can influence the climate by regulating root secretions to reduce nitrogen loss and the carbon footprint, and also by recruiting beneficial microorganisms that enhance the ability to suppress pathogens [159]. The warming of the climate and N₂ and CO₂ also alter crop–soil interactions [160], while elevated CO₂ concentration indirectly affects the size of the population of microbes in the soil [161] and its diversity [162] through physicochemical properties such as soil temperature, moisture, and pH, as well as crop root morphology and root secretions [163]. Microorganisms can inhibit crop diseases by fixing nitrogen [164] and promoting crop growth [165,166], or even by changing the structure of the microbiome from a dysfunctional to a healthy state by altering environmental conditions [167]. The release of metabolites from maize roots affects the composition of root-associated microbiota [168], and the excretion of taxifolin by tomato plants serves as a catalyst for the attraction and recruitment of beneficial bacterial species [169]. The disadvantages of ecological regulation are the complexity of its application and the long time it takes to obtain results.

Effective responses to crop fungal diseases under global climate change conditions require a combination of control measures to realize the dynamic balance of crop–biology–soil, and the capacity of various control measures to enable adaptation to climate change should be fully exploited, such as improving the physicochemical properties of soil and reducing the relative abundance of soil pathogens by performing supplementation with soil conditioning agents, such as biochar, when microbial fungicides are applied [170]. Immobilization of microorganisms in materials can also reduce their exposure to extreme climatic conditions and improve environmental resilience [171]. Meanwhile, the intensive use of multidisciplinary tools, such as varietal performance prediction [172], can help develop new varieties that can adapt to climate change. Moreover, microbial pesticides or nanobiopesticides derived from microorganisms, such as bacteria, fungi, viruses, protozoa, and algae, are being intensively explored to control crop diseases. In this context, environmentally friendly and green control measures also have great potential, such as the application of biopesticides prepared from wood mold for the control of root rot of chickpea [173] and the application of biocontrol agents such as a *Trichoderma* and chitosan-polyethylene glycol (PEG) hybrid system for seed treatment [174]. Nanobiopesticides have beneficial physicochemical properties, such as a small particle size, easy reactivity, large surface area, clear biological interactions with crops, and clear transport and fate in the environment (Table 4).

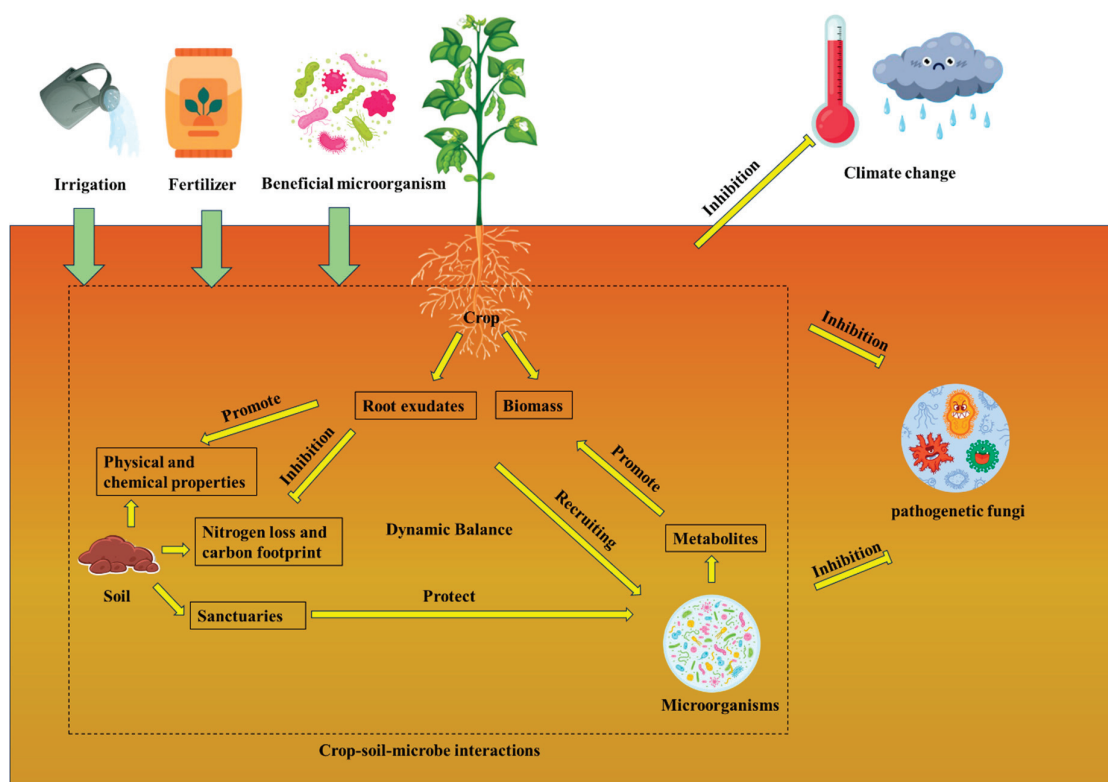


Figure 2. Dynamic equilibrium between crop–biology–soil.

Table 4. Control of crop fungal diseases under climate change.

Preventive and Curative Measures	Principles of Prevention and Treatment	Advantages	Disadvantages	Reference
Breeding for disease resistance	Targeted selection or alteration of certain genotypes to produce new varieties of crops that are resistant to disease	One of the most effective and economical measures	High-quality resistance results in the targeted selection of pathogens	[126–128]
Agronomic measures	Fertilization, irrigation, and so on to create environmental conditions suitable for the growth and development of vegetables and the survival and reproduction of beneficial organisms, but not conducive to the occurrence of pathogenic fungi	Improve the physical and chemical properties of the soil to provide a good growing environment for crops	High upfront costs, and labor-intensive and time-consuming process	[129–131,134–136]
Chemical control	Chemical pesticides kill pathogenic fungi	By far the most commonly used control method	Pathogen resistance, environmental risks	[137–152]
Biological control	Use of organisms such as crops, insects, and microorganisms to limit diseases caused by pathogens	A green, healthy, and promising approach	Colonization by free microorganisms is difficult, and the preventive effect is unstable in extreme climate conditions such as drought and high temperature	[153–157]
Ecological regulation	Provide a favorable environment for healthy crop growth and maintain crop–biology–soil dynamic balance	Maintaining a dynamic crop–biology–soil balance	the complexity of its application and long time to obtain results.	[158–170]

5. Summary and Outlook

With advancement in global climate change, extreme climatic events, such as high temperatures, droughts, and floods, are becoming more frequent and more severe. Meanwhile, outbreaks of fungal diseases of crops are becoming more frequent and serious and disrupt-

ing the dynamic balance among crops, organisms, and the soil. Intensifying climate change necessitates the development of new data acquisition technologies and diagnostic methods, such as RS, combined with artificial intelligence coupling technology, to collect and analyze disease data in an accurate, timely, and efficient manner on the basis of existing methods for monitoring and diagnosing crop fungal diseases. After determining the areas affected by disease, the ecological regulation can be used to organize the implementation of ecological control measures and traditional control measures, apply the control technology in the context of climate change, maintain the health of the soil and crop, protect the diversity of the overall community of microorganisms, and slow down the harm caused by crop fungal diseases in association with climate change. The ecological management of crop–biological–soil dynamics is considered to be an effective measure to combat fungal diseases in the future, and this new concept promotes the green development of agriculture and mitigates the damage caused to crops by fungal diseases under extreme climatic conditions.

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Article

Influence of Nitrogen Fertilisation Level and Weather Conditions on Yield and Quantitative Profile of Anti-Nutritional Compounds in Grain of Selected Rye Cultivars

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Abstract: Cultivar, habitat conditions and agrotechnology have an influence on the yield and chemical composition of rye grain. The main anti-nutritional substances present in rye grain include alkylresorcinols, water-soluble pentosans and trypsin inhibitors. The aim of this study was to determine the variability in yield and the concentration of anti-nutritional compounds in the grain of selected winter rye cultivars in relation to nitrogen fertilisation levels and weather conditions. Field studies were conducted at the Experimental Station of IUNG-PIB in Osiny (Poland) in two growing seasons (2018/2019 and 2019/2020). The experiment was located on pseudo-polylic soil using the randomised sub-block method in three replications. The first factor of the experiment was the level of nitrogen fertilisation (0, 70 and 140 kg N·ha^{−1}) and the second was the population (Dańkowskie Skand, Piastowskie) and hybrid (KWS Vinetto, SU Performer) winter rye cultivars. The study showed that the yield of winter rye depended on the genotype and the level of nitrogen fertilisation. The hybrid cultivars yielded 17.9% higher in relation to the population cultivars. The content of anti-nutritional compounds in rye grain depended significantly on genotype, level of nitrogen fertilisation and weather conditions. The reason for the higher synthesis of anti-nutrients in rye grain was the stressful weather conditions occurring in the 2019/2020 season. Nitrogen fertilisation influenced the content of alkylresorcinols, water-soluble pentosans and trypsin inhibitor activity in grain. The interaction of cultivar and fertilisation was also found to shape the content of the aforementioned anti-nutrients.

Keywords: alkylresorcinols; cultivars; grain yield; nitrogen fertilisation; pentosans; rye; trypsin inhibitors

1. Introduction

Rye (*Secale cereale* L.) is a cereal grown in Europe in a large area of the so-called “rye belt”, which includes countries such as Germany, Poland, Ukraine and Belarus. In recent years, rye production in the European Union has been around 8 million tonnes per year [1]. In the food industry, rye grain serves mainly as a raw material for the production of different types of flour from which bread (rye, mixed) is made [2]. Products made from rye flour, especially whole-grain flour, are characterised by their high nutritional and health-promoting values [3]. Ongoing breeding work focused on improving grain yield and quality further increases the potential for the use of rye grain in both human and animal

nutrition [4]. The breeding of new cultivars aims to increase grain yield and quality by using cultivars with stable yields under variable climatic conditions [5]. A particular direction of progress in breeding cultivars with high yield potential is to obtain hybrid cultivars of rye [6,7]. The heterosis effect occurring in hybrid cultivars allows for higher grain yields resulting from higher ear density [8–10]. Hybrid cultivars, as a result of transgressive segregation of alleles, may show a different grain chemical composition [11]. Higher grain yield and better grain quality influence the increased interest in the cultivation of hybrid cultivars. There is considerable interest in the cultivation of hybrid rye cultivars in Western European countries in particular, which account for 81% of total rye grain production, with population cultivars accounting for the remainder [12]. The main factors in favour of rye cultivation are its low soil requirements, resistance to low winter temperatures and relatively high grain yield. Yield and its quality are determined by weather conditions during the growing season and appropriately selected agrotechnics, including the level of nitrogen fertilisation, as well as by the cultivar [13,14].

A significant proportion of rye grain is used in the feeding of various animal species; mainly pigs and poultry [4]. However, a limitation to its wider use is its content of anti-nutritional substances that have a negative impact on animal health and development. These include alkylresorcinols, water-soluble pentosans and trypsin inhibitors. However, due to the varietal variation associated with the content of these substances, the grain of some modern rye cultivars may have a higher feed value [15]. The rye grain of hybrid cultivars can be used in poultry feed at a higher proportion than population cultivars [16–19].

The main anti-nutritional substances found in rye grain include alkylresorcinols, non-starch polysaccharides and trypsin inhibitors [19]. Alkylresorcinols are a group of phenolic lipids. Most of these compounds are located in the central parts of the fruit and seed coat. These compounds were found in the largest amount in rye grain ($360\text{--}2180\text{ mg}\cdot\text{kg}^{-1}$), followed by triticale grain ($294\text{--}1145\text{ mg}\cdot\text{kg}^{-1}$) and wheat ($268\text{--}943\text{ mg}\cdot\text{kg}^{-1}$) [20]. Literature data indicate that alkylresorcinols interact with other compounds present in grain to reduce animal weight gain [21]. Feeding animals cereal grain from which alkylresorcinols have been extracted increases production effects [22]. Compared to other cereals, rye grain contains the highest amount of these compounds [23]. The high water-binding capacity of NSP and the resulting swelling hinders the penetration of digestive contents by enzymes that hydrolyse starch, proteins and fats. Compared to other cereals, rye grain contains the highest amount of these compounds, which swell in the digestive tract, causing reduced feed intake, poorer utilisation of the nutrients and energy contained in the feed, and ultimately lower body weight [24]. The only method to neutralise their anti-nutritional effect is to add xylanase enzymes to the feed [25]. Climate change and the resulting requirements to reduce the environmental impact of feed production are the main challenges in animal nutrition. In the case of wheat, the grain of which is widely used for the production of feed, rye uses a natural solution, primarily water and phosphorus, and its production produces a carbon footprint [26]. However, a large amount of non-starch polysaccharides used in rye grain may increase intestinal fermentation, which may affect the intestinal life, especially in young monogastric animals, such as piglets and chickens for fattening. Among the available anti-nutritional agents against the action of pentosans and alkylresorcinols are geese, in which hybrid rye grains are fed with a nutritional value comparable to oats [27]. In older animals, the anti-nutritional effect of alkylresorcinols and water-soluble pentosans is no longer as strong, and may even have an impact on health by acting prebiotically. The strongest anti-nutritional effect in animals, both young and adult, involves inhibiting trypsins. They inhibit one of the proteolytic enzymes (proteases) by use, which causes it to work more intensively and cause an increase in mass [28].

Higher trypsin inhibitor activities are found in rye grain than in the grains of other cereal types (wheat and triticale). These compounds are found in both endosperm and embryos. Their activity is associated with low molecular weight cereal proteins, which include albumin and globulins [29]. The adverse effects of inhibitors on the animal body are mainly due to a reduction in the activity of digestive enzymes and a decrease in digestibility,

as well as the utilisation of nutrients, especially protein, leading to a reduction in animal weight gain [30].

The amount of anti-nutritional compounds in cereal grain depends mainly on the type, species and variety of cereal. However, it is also largely shaped by climatic, soil and agrotechnical conditions, as well as by the time and conditions of grain storage after harvest [31,32]. Among the agrotechnical factors, nitrogen fertilisation has the greatest impact on the yield and chemical composition of grain. The use of nitrogen by cereals depends on genetic and environmental factors [33]. Research carried out so far shows that rye varieties use increasing doses of nitrogen unequally [34]. Individual varieties differ significantly in terms of the rate of growth of vegetative mass and nitrogen accumulation, especially with abundant fertilisation with this element. The uptake and remobilization of nitrogen by cereal plants is largely influenced by weather conditions during the growing season. Climate changes, and especially droughts, cause cereal plants to less effectively use nitrogen more, which leads to lower yields, and therefore the concentration of nitrates in the dry matter of grain is higher than in wet years [35,36]. The few available pieces of literature on this subject [17,37,38] indicate that nitrogen fertilisation affects the concentration of anti-nutritional compounds in cereal grains, such as alkylresorcinols, pentosans and the activity of trypsin inhibitors. Safar-Noori et al. [39] showed that, as the level of nitrogen fertilisation increases, the content of protein and pentosans in wheat grain increases. The literature on the subject lacks research results on the influence of the level of nitrogen fertilisation on the synthesis of anti-nutritional compounds in the grain of population and hybrid varieties of winter rye; however, the obtained research results will fill the gap regarding this issue. The research hypothesis assumed that both the nitrogen fertilisation dose and weather conditions during the growing season would differentiate the content of anti-nutritional compounds in the grain of the tested winter rye varieties.

The aim of the study was to determine the yield and variability in the concentration of anti-nutritional compounds in the grain of selected winter rye cultivars in relation to the level of nitrogen fertilisation and weather conditions.

2. Materials and Methods

2.1. Site Characteristic, Experimental Design, and Agronomic Practices

The field experiment was conducted in the growing seasons 2018/2019 and 2019/2020 at the Experimental Station in Osiny (51°27' N; 22°2' E), belonging to the Institute of Soil Science and Plant Cultivation—State Research Institute (IUNG-PIB) in Pulawy (Poland). The experiment was located on pseudo-loamy soil, good wheat complex, quality class IIIa and IIIb. The soil of pH 6.5 contained 181 mg K·kg⁻¹ and 173 mg P·kg⁻¹. Before applying nitrogen fertilisation, the content of mineral nitrogen (NH₄⁺, NO₃⁻) in the soil was determined in early spring, which in 2019 was 39.6 kg N·ha⁻¹ and 32.6 kg N·ha⁻¹ in 2020, respectively. The two-factor experiment was set up using the randomised sub-block method in triplicate. The sowing of rye was carried out between 20th–30th of September, and the crop was cultivated after winter oilseed rape in a crop rotation with 75% share of cereals in the sowing structure. The experiment was conducted with a ploughing system. After harvesting the forecrop, straw was shredded and ploughed to a depth of 8–10 cm, then harrowed with a heavy harrow, followed by pre-sowing ploughing to a depth of 20–22 cm.

The first factor of the experiment was the level of nitrogen fertilisation: 0, 70 and 140 kg N·ha⁻¹. A nitrogen dose of 70 kg N·ha⁻¹ was applied at two dates: 40 kg N·ha⁻¹ in spring at the start of vegetation and 30 kg N·ha⁻¹ at the stalk shooting stage (BBCH 31). A nitrogen dose of 140 kg N·ha⁻¹ was applied at three dates: 70 kg N·ha⁻¹ in spring at the start of vegetation, 50 kg N·ha⁻¹ at the stalk shooting stage (BBCH 31) and 20 kg N·ha⁻¹ at the earing stage (BBCH 57). Nitrogen was applied in a form NH₄NO₃.

The second factor of the experiment was the winter rye cultivars. Two population cultivars were included, Dańkowskie Skand and Piastowskie, and two hybrid cultivars, KWS Vinetto and SU Performer.

Dańkowskie Skand—Hodowla Danko—cultivated since 2017 <https://danko.pl/en/odmiany-type/dankowskie-skand-2/> (accessed on 21 February 2024).

Piastowskie—Poznańska Hodowla Roślin—cultivated since 2017 <https://phr.pl/produkty/zboza-ozime/zyto-ozime-piastowskie/> (accessed on 21 February 2024).

KWS Vinetto—KWS cultivated since 2017—<https://www.kws.com/pl/pl/produkty/zboza/zyto/przeglad-odmian-zyto/kws-vineto/> (accessed on 21 February 2024).

SU Performer—Saaten Union cultivated since 2014 <https://www.saaten-union.pl/zyto/zyto-ozime-hybrydowe/su-performer/> (accessed on 21 February 2024).

The area of the experimental plot to be harvested was 20.0 m². The population rye was sown at 2.5 million grains·ha^{−1}, and hybrid rye at 2.0 million grains·ha^{−1}. Pre-sowing mineral fertilisation was applied in the form of superphosphate at a rate of 60 kg P₂O₅·ha^{−1} and potassium salt at a rate of 90 kg K₂O·ha^{−1}. Protection against diseases, pests and weeds was carried out according to the integrated method of reducing weeds and disease and pest pathogens after exceeding the threshold of harmfulness. Immediately after harvest, the grain yield per unit area was assessed.

2.2. Meteorological Conditions

The course of weather conditions was described on the basis of monthly mean values of air temperature and total precipitation in comparison with climatic norms and mean values for the period 1981–2010. Meteorological data were obtained from the Agro-meteorological Station located near the experimental fields at the Experimental Station IUNG–PIB in Osiny ($\varphi = 51.469$ N, $\lambda = 22.052$ E). The years in which the study was conducted differed in terms of thermal conditions and precipitation amounts. The 2018/2019 growing season was characterised by lower precipitation, while at the same time there were large temperature fluctuations (Figure 1a). During the autumn period (September to December), precipitation was average and temperatures were slightly higher compared to multi-year averages (1980–2010). An exceptionally warm period was February (3.4 °C) and March (4.5 °C), in these months the average temperature was 4.1 and 3.2 °C higher, respectively, compared to the multi-year period (1981–2010). In May, there was a change in the trend of thermal conditions. After an exceptionally warm spring, May was cool (12.9 °C) and characterised by high precipitation (86.1 mm). June saw another change in weather trends; it was a hot month with low rainfall. The temperature in June was 21.7 °C, 5.1 °C higher than the multi-year period (1981–2010), while precipitation was low at 38.7 mm, which was only 62% of normal.

The second growing season 2019/2020 from autumn to spring (September to March) was characterised by higher air temperatures compared to the 1981–2010 multi-year average (Figure 1b). A particularly outstanding period was winter (December to February), which was very warm with high precipitation. The temperature during this period was: in December 3.1 °C, in January 1.8 °C and in February 3.4 °C, compared to the multi-year average (1981–2010) it was higher by, respectively: 4.0, 4.1 and 4.9 °C. In addition, the period was characterised by high precipitation totalling 153 mm, which was 180% of the precipitation compared to the multi-year average (1981–2010). A change in the trend of weather conditions occurred in March and April. During this period, precipitation totals were very low, amounting to only 34 mm, which was 54% of the precipitation compared to the multi-year average (1980–2010). After a period with low precipitation, the next two months (May and June) were characterised by high precipitation. In May, the precipitation was 112 mm, while in June it was even higher, at 189 mm; compared to the multi-year period, precipitation was 101 and 206% higher, respectively. In contrast, thermal conditions in these months were different. May was cold (11.1 °C), while June was warm (18.4 °C). Compared to the multi-year period (1981–2010), the temperature in May was 2.8 °C lower, while in June it was 1.8 °C higher.

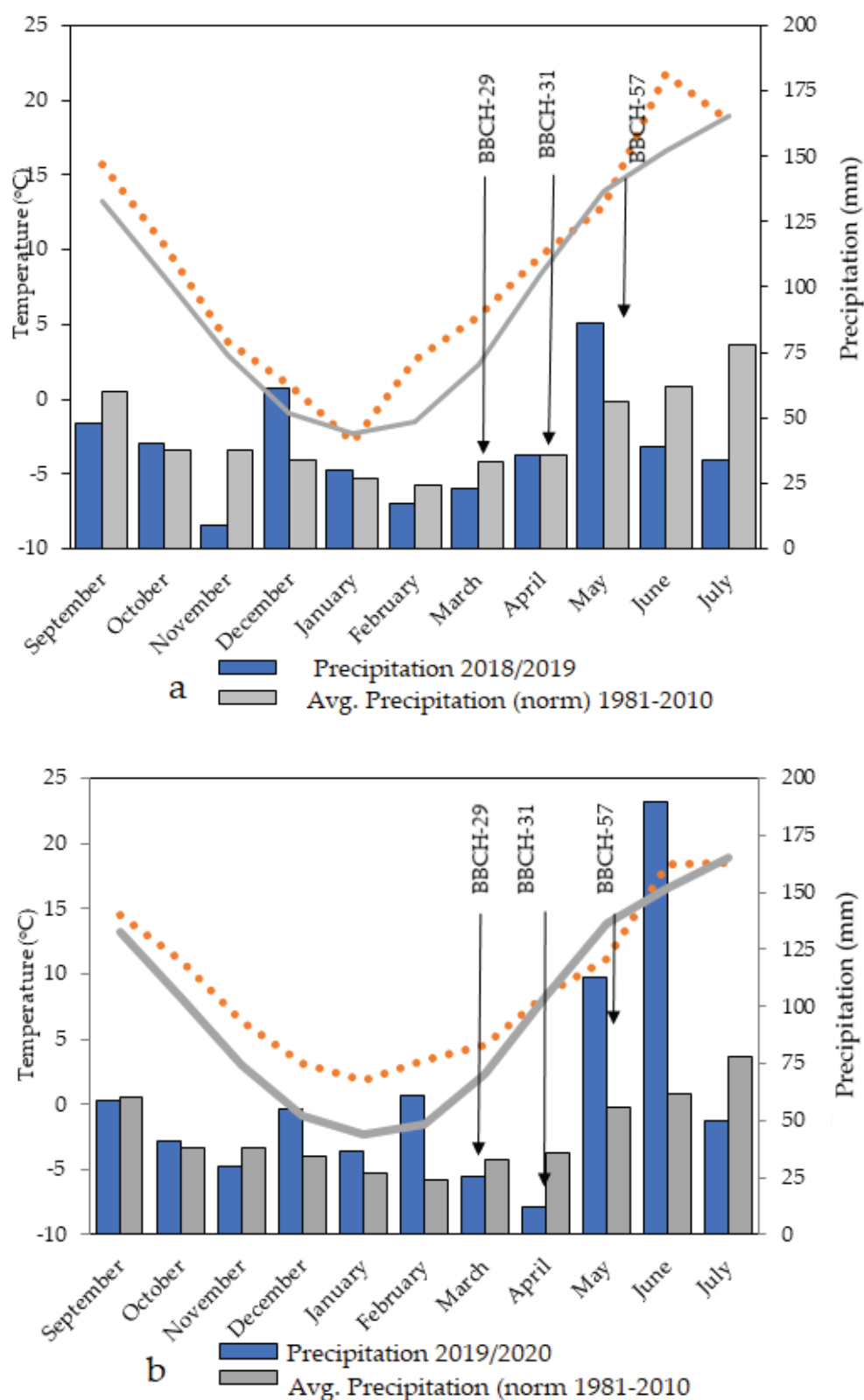


Figure 1. (a) Monthly air temperature (°C) and monthly of precipitation (mm) in years 2018/2019. The arrow in the graph indicates the date of nitrogen application in a given BBCH phase at the dose indicated in Section 2.1. (b) Monthly air temperature (°C) and monthly of precipitation (mm) in years 2019/2020. The arrow in the graph indicates the date of nitrogen application in a given BBCH phase at the dose indicated in Section 2.1.

2.3. Chemical Analyses

2.3.1. Determination of Alkylresorcinol Content

The analysis was performed by the spectrophotometer method [40]. Grains were ground to a particle size of <0.5 mm. Alkylresorcinols were extracted with acetone from the weighed amount of 1 g in the time of 3 h at 55 °C in a water bath. The content of alkylresorcinols was determined using acetone extracts after developing a colour reaction with the two-phase p-nitroaniline and measuring the absorbance of the coloured solution at 435 nm. For preparing the calibration curve, the orcinol was used.

2.3.2. Determination of Soluble Pentosans

The orcinol-hydrochloric acid method of Albaum and Umbreit [41] for pentose determination was used. It consisted of heating in boiling water for 30 min a solution containing 3 mL of pentose, 3 mL of 0.1% ferric chloride in concentrated hydrochloric acid, and 0.3 mL of 1.0% orcinol in 100% ethanol, cooling, and determining absorbance at 670 nm.

2.3.3. Determination of the Activity of Tripsin Inhibitors

Tripsin inhibitors were determined by the method of PN-EN ISO 14902 [42], which were extracted from the sample at pH 9.5. The residual trypsin activity was measured by adding benzoyl-L-arginine-p-nitroanilide (L-BABA) as a substrate. The amount of p-nitroanilide released was measured spectrometrically.

2.4. Statistical Analysis

Results recorded in the course of conducted chemical analyses were subjected to statistical analysis with the use of R 4.3.0 [43]. In order to compare the grain yield and contents of alkylresorcinol, soluble pentosans and tripsin inhibitors between the cultivars and N fertilisation effects, we used two-way ANOVA as a post hoc test, and Tukey's multiple comparison procedure was also used, with identical letters denoting a lack of differences at the significance level $\alpha = 0.05$. The assumption of normality of the layouts was checked using the QQ Plots for residuals and the homogeneity of variances using the Levene's test. Moreover, a principal component analysis (PCA) was used to evaluate the relationship between study traits and factors.

3. Results and Discussion

Winter rye yields depended on the cultivar and nitrogen fertilisation rate. In both 2019 and 2020, the highest grain yield was obtained from hybrid cultivars. On average, hybrid cultivars yielded 17.9% higher than population cultivars during the study years. In 2019, the hybrid cultivar KWS Vinetto had the highest yield ($8.44 \text{ t} \cdot \text{ha}^{-1}$), and in 2020 the hybrid cultivar SU Performer ($8.33 \text{ t} \cdot \text{ha}^{-1}$) had the highest yield (Figure 2). Literature data [44–46] also indicate that hybrid winter rye cultivars achieve higher grain yields compared to population cultivars. In a study by Latusek and Bujak [44], the grain yield of hybrid cultivars was 17.7% higher, while in a study by Wyzińska et al. [45] it was 11.2% higher in relation to population cultivars. Maciorowski et al. [34] found that hybrid cultivars yielded 9% higher compared to population cultivars. Also, a comparative study by Szuleta et al. [46] on 24 population and hybrid rye cultivars showed a yield advantage for the hybrid cultivars, especially in years with less favourable weather conditions. The higher grain yield of hybrid rye cultivars shown in both years of the study compared to population cultivars may have been due to the higher tolerance of these cultivars to lower rainfall, especially in March and April, which were lower than the multi-year average. Hybrid cultivars characterised by higher tillering and vegetative mass structure provided more stable yields than population cultivars [47]. The reduction in rye grain yields as a result of unstable weather conditions in spring could reach up to 17.1% [48,49].

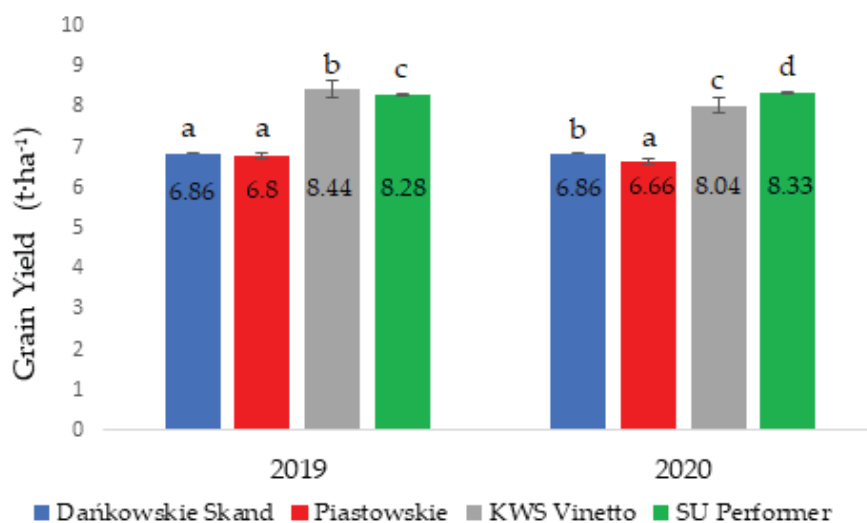


Figure 2. Grain yield of winter rye depending on the cultivar (average for a change). a, b, c, d—the same letters indicate no significant differences at significance level $\alpha = 0.05$.

A significant effect of nitrogen fertilisation level on winter rye yield was found. In 2019, a significant increase in grain yield occurred at a dose of $70 \text{ kg N} \cdot \text{ha}^{-1}$, and further increases in nitrogen dose did not cause a significant increase in grain yield. In contrast, in 2020, grain yield increased significantly alongside the growing nitrogen fertilisation rates. The highest rye grain yield was obtained at the application of $140 \text{ kg N} \cdot \text{ha}^{-1}$, and it was 4.6% higher with respect to the dose of $70 \text{ kg N} \cdot \text{ha}^{-1}$ (Figure 3). Latusek and Bujak [44] and Dopierała et al. [50], comparing rye yield at two agrotechnical levels, observed a positive response of both hybrid and population cultivars to higher levels of nitrogen fertilisation. In contrast, a study by Noland [51] showed that a significant increase in rye yield occurred only up to an application rate of $67 \text{ kg N} \cdot \text{ha}^{-1}$. Population cultivars, however, showed a weaker yield response to nitrogen fertilisation than hybrid cultivars [52]. Szuleta et al. [53] found no significant differences in the grain yield of rye population cultivars as a result of doubling the nitrogen dose. The efficiency with which rye utilises the supplied nitrogen dose depends on weather conditions, especially the amount of rainfall during the growing season, but also the content of organic matter, which accumulates in the water reserve [48,54,55].

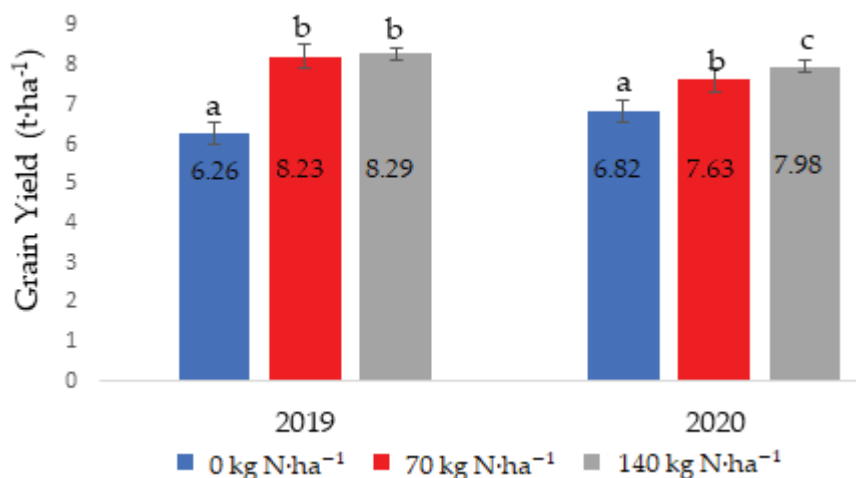


Figure 3. Grain yield of winter rye depending on the dose of nitrogen (average for nitrogen fertilisation). a, b, c—the same letters indicate no significant differences at significance level $\alpha = 0.05$.

It was found that the content of the tested anti-nutritional substances in rye grain was significantly influenced by the cultivar, nitrogen fertilisation rate and weather conditions during the period of field cultivation, i.e., 2018–2020.

The differences in the content of anti-nutritional compounds in rye grain from the different harvest years gave us an incentive to analyze the weather conditions during the different growing seasons (Figures 2 and 3). Significantly, the grain harvested in 2019 had the lowest content of anti-nutrients (Table 1). In rye grain from 2019, the average content of alkylresorcinols was $533.0 \text{ mg}\cdot\text{kg}^{-1}$, soluble pentosans was 1.29% d.m. and trypsin inhibitor activity was $1.19 \text{ mg}\cdot\text{g}^{-1}$. A higher content of anti-nutritional compounds was found in rye grain from 2020. The average content of alkylresorcinols was $658.2 \text{ mg}\cdot\text{kg}^{-1}$, water-soluble pentosans was 1.46% of d.m. and trypsin inhibitor activity was $1.32 \text{ mg}\cdot\text{g}^{-1}$. Increased synthesis of anti-nutritional compounds was ascribed to stress conditions during the growing season. In the 2019/2020 growing season, the stress was caused by high precipitation in the months of May and June, which was well above normal, resulting in high levels of fungal diseases in the rye plantation. Previous studies [17] have also shown that the content of anti-nutritional substances in rye grain (alkylresorcinols, water-soluble pentosans, trypsin inhibitors) is strongly influenced by weather conditions during the plant growing season. Increased synthesis of the aforementioned anti-nutritional compounds in the grain occurred when stress conditions for plant development occurred during the growing season. The highest levels of anti-nutritional substances were synthesised when plants experienced stress caused by a shortage of rainfall during their intensive growth period (at the stalk shooting stage), as well as too much rainfall in May and June (at the earing and flowering stages). On the other hand, Jaśkiewicz and Szczepanek [38], investigating the content of alkylresorcinols in winter triticale grain, found that lower accumulation of these compounds was favoured by limited precipitation at the stalk shooting stage and excess precipitation during grain maturation. Similarly, Bellato et al. [55] showed that durum wheat grain contained more alkylresorcinols when there was less rainfall during the growing years. Weather conditions during the growing season also affect the accumulation of water-soluble pentosans in rye grain. Less rainfall during the growing season favours higher content in rye grain [49,53]; in winter wheat, on the other hand, a higher accumulation of pentosans in the grain is favoured by a lower temperature at the grain pouring stage [56]. Marentes-Culma and Coy-Barrera [57] showed that grain of the same cultivar of triticale from cultivation under different environmental and weather conditions contains different amounts of alkylresorcinols.

The grain of the winter rye cultivars tested was characterised by different contents of anti-nutritional compounds. The highest content of alkylresorcinols in rye grain in both 2019 and 2020 was found in the grain of the KWS Vinetto hybrid cultivar, reaching 555.2 and $674.2 \text{ mg}\cdot\text{kg}^{-1}$, respectively (Figure 4A). In 2019, the lowest content of alkylresorcinols was found in the grain of the rye population cultivars Dańkowskie Skand ($510.3 \text{ mg}\cdot\text{kg}^{-1}$) and Piastowskie ($532.0 \text{ mg}\cdot\text{g}^{-1}$). In 2020, the least amount of these compounds was contained in the grain of the hybrid cultivar SU Performer ($643.3 \text{ mg}\cdot\text{kg}^{-1}$). On average, over the years of the study, the grain of the KWS Vinetto hybrid cultivar had the highest content of alkylresorcinols ($614.70 \text{ mg}\cdot\text{kg}^{-1}$) (Table 1). Similarly, other authors [48,49,54] have shown that the content of alkylresorcinols in cereal grain is mainly determined by a genetic factor. Grabiński et al. [17] showed that, during the three-year study period, the highest alkylresorcinol content was found in grain of the hybrid cultivar Visello (mean $563 \text{ mg}\cdot\text{kg}^{-1}$) and the lowest in the grain of the hybrid cultivar Brasetto (mean $535 \text{ mg}\cdot\text{kg}^{-1}$). A study by Kulawianek et al. [58] showed that the highest accumulation of these compounds was in the grain of the hybrid cultivar Gradan ($1152 \text{ mg}\cdot\text{kg}^{-1}$ d.m.) and the lowest in the grain of the population cultivar Amilo ($1058 \text{ mg}\cdot\text{kg}^{-1}$ d.m.). Studies by Targońska-Karasek et al. [59] also showed the influence of the genetic factor on the synthesis of alkylresorcinols, while the lowest potential for alkylresorcinol synthesis was demonstrated by the population cultivar Dańkowskie Złote in comparison to the hybrid cultivar Daniello and the population cultivar L 318. Jaśkiewicz and Szczepanek [38] showed that, for both winter and spring

triticale, there are varietal differences in the content of alkylresorcinols in grain. Similar relationships were found for winter wheat [57,59–61].

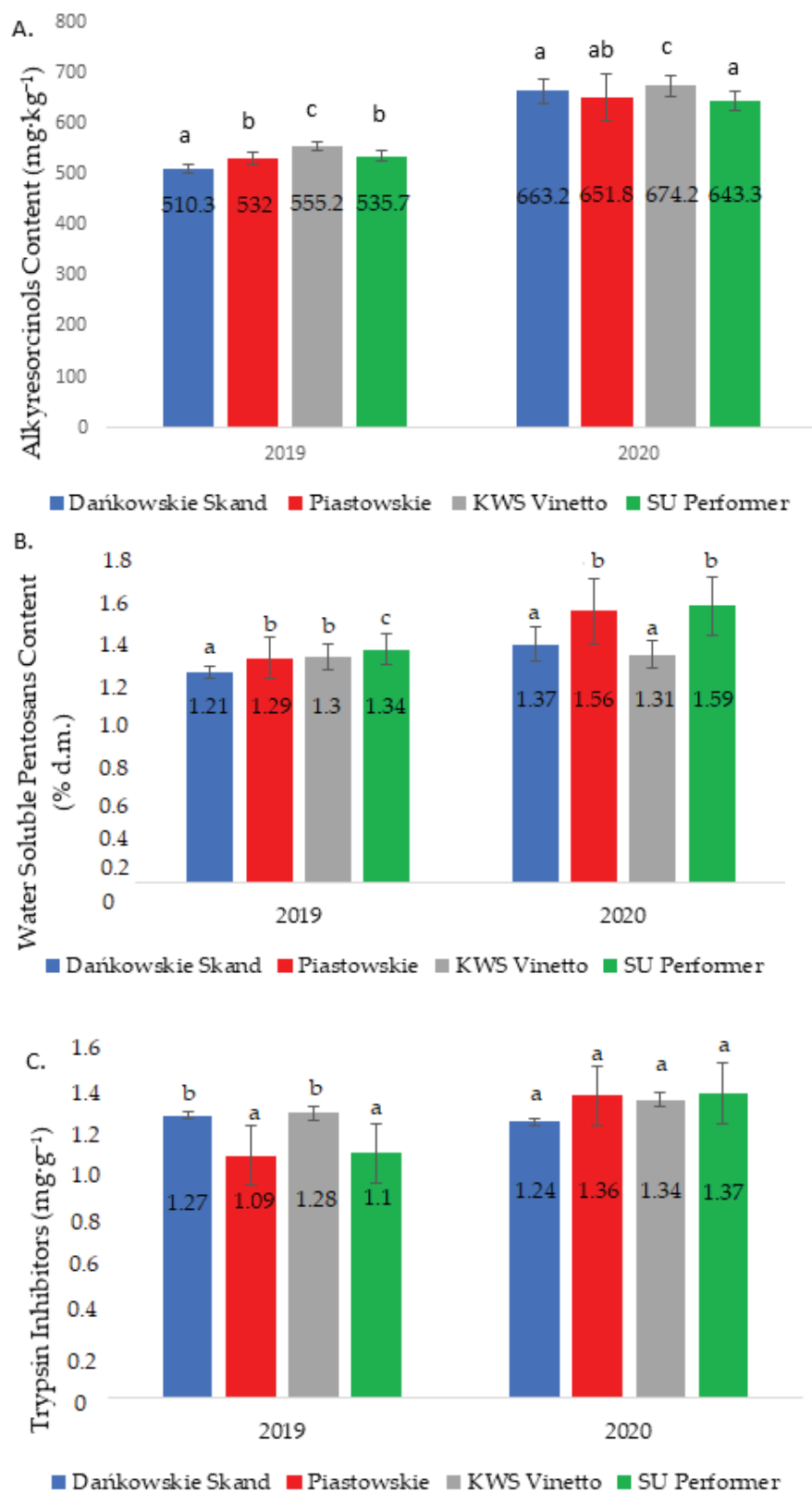


Figure 4. Content of selected anti-nutritive components: alkylresorcinols (A), water-soluble pentosans (B) and trypsin inhibitors (C) in grain cultivars in 2019–2020. a, b, c—the same letters indicate no significant differences at significance level $\alpha = 0.05$.

Table 1. Compounds of selected anti-nutritional substances in the grain of winter rye varieties depending on the growing season, variety and nitrogen fertilisation.

Specification	Alkylresorcinol Content (mg·kg ⁻¹)	Water-Soluble Pentosans Content (% d.m.)	Activity of Trypsin Inhibitors (mg·g ⁻¹)
Growing season			
2018/2019	533.29 ^a	1.28 ^a	1.23 ^a
2019/2020	661.79 ^b	1.46 ^b	1.46 ^b
Cultivar			
Dańkowskie Skand	586.75 ^a	1.29 ^a	1.26 ^a
Piastowskie	591.90 ^{ab}	1.42 ^{ab}	1.23 ^a
KWS Vinetto	614.70 ^b	1.31 ^{ab}	1.31 ^a
SU Performer	588.00 ^{ab}	1.47 ^b	1.24 ^a
Nitrogen Fertilisation (kg·ha⁻¹)			
0	575.20 ^a	1.41 ^a	1.28 ^a
70	610.55 ^b	1.38 ^a	1.34 ^b
140	601.45 ^a	1.31 ^a	1.14 ^a

^{a, b}—the same letters indicate no significant differences at significance level $\alpha = 0.05$.

The content of water-soluble pentosans, like alkylresorcinols, was shaped by the genetic factor. In 2019, the content of water-soluble pentosans in rye grain ranged from 1.21 to 1.34% d.m., and in 2020 from 1.31 to 1.56% d.m. (Figure 4B). In 2019, the most water-soluble pentosans were contained in the grain of the hybrid cultivars KWS Vinetto and SU Performer. On the other hand, in 2020, the highest concentration of these compounds was found in the grain of the hybrid cultivar SU Performer and the population cultivar Piastowskie. On average, over the years of the study, the grain of the SU Performer hybrid variety had the highest content of soluble pentosans (1.47% d.m.) (Table 1). Studies by other authors [17,55,62] also showed that the content of soluble pentosans in rye grain depends on the cultivar. Buksa et al. [37] showed that there is a very high variation in the content of water-soluble pentosans in the grain of population cultivars of winter rye. According to Grabiński et al. [17], the most water-soluble pentosans are contained in the grain of hybrid rye cultivars. However, Kulichova et al. [62] showed no significant differences between rye cultivars in the content of water-soluble pentosans in grain. The varietal factor also has a strong influence on the content of pentosans in wheat and barley grain [50].

In 2019, the activity of the trypsin inhibitors in rye grain was shaped by a genetic factor. The grain of the population cultivar Dańkowskie Skand (1.27 mg·g⁻¹) and the hybrid cultivar KWS Vinetto (1.27 mg·g⁻¹) had significantly higher activity of these substances than the grain of the population cultivar Piastowskie (1.09 mg·g⁻¹) and the hybrid cultivar SU Performer (1.10 mg·g⁻¹) (Figure 4C). In 2020, there were no significant differences in the activity of trypsin inhibitors in the grain of the rye cultivars tested; there was only a trend towards lower activity in the grain of the population cultivar Dańkowskie Skand. Over the years, it was shown that the grain of the KWS Vinetto hybrid cultivar was characterized on average by the highest activity of trypsin inhibitors (1.31 mg·g⁻¹) (Table 1). In a study by Grabiński et al. [17], it was shown that the activity of trypsin inhibitors depended on the genetic factor. The authors obtained the highest trypsin inhibitor activity (2.80 mg·g⁻¹) in the grain of the hybrid cultivar Visello, and the lowest (2.01 mg·g⁻¹) in the grain of the population cultivar Dańkowskie Diament. Kulichova et al. [62], in examining 19 rye cultivars, showed that most of them had similar trypsin inhibitor activity, but also found the occurrence of two cultivars with significantly lower activity of this parameter, indicating genetic variation. In addition, Simonetti et al. [63] showed that the activity of trypsin

inhibitors in wheat grain depends on the species and cultivar, and also that environmental conditions also have a significant effect.

Our own research has shown the interaction of cultivar and nitrogen fertilisation rates in shaping the content of anti-nutrients in winter rye grain (Figure 5A–F). In 2019, a significant increase in the content of alkylresorcinols was obtained in the grain of the population cultivars Dańkowskie Skand and Piastowskie, as well as the hybrid cultivar KWS Vinetto, when a dose of $70 \text{ kg N}\cdot\text{ha}^{-1}$ was applied; further increasing the dose to $140 \text{ kg N}\cdot\text{ha}^{-1}$ did not result in an increase in the concentration of these substances in the grain (Figure 5A). In contrast, in the case of the hybrid cultivar SU Performer, grain with the highest alkylresorcinol content was obtained using a fertilisation rate of $140 \text{ kg N}\cdot\text{ha}^{-1}$. In 2020, there was no significant effect of increasing the nitrogen fertilisation rate on the content of alkylresorcinols in the grain of the population cultivar Dańkowskie Skand (Figure 5B). In contrast, the population cultivar Piastowskie and the hybrid cultivar KWS Vinetto reacted with a significant increase in the content of these compounds when fertilized with $140 \text{ kg N}\cdot\text{ha}^{-1}$. In the grain of the hybrid cultivar SU Performer, the highest content of alkylresorcinols was found after fertilisation at a dose of $70 \text{ kg N}\cdot\text{ha}^{-1}$. Literature reports indicate that cereal cultivars show a differentiated response in alkylresorcinol content to increasing doses of nitrogen fertilisation [17,53,61]. Grabiński et al. [17] showed that an increase in the intensity of production technology, including nitrogen fertilisation, caused a significant increase in alkylresorcinol content by 7%. According to Jaśkiewicz and Ochman [64], nitrogen fertilisation at a dose of $100 \text{ kg}\cdot\text{ha}^{-1}$ favoured AR (alkylresorcinols) accumulation in spring triticale grain, especially under conditions of higher rainfall in April and May. On the other hand, under conditions of limited rainfall at the tillering stage and the beginning of stalk shooting, AR content in grain was similar at both fertilisation levels of 70 and $100 \text{ kg N}\cdot\text{ha}^{-1}$. Takač et al. [65], found that spelt wheat grain contained more alkylresorcinols in organic cultivation compared to conventional cultivation where increased mineral nitrogen fertilisation is applied.

In 2019, the highest concentration of water-soluble pentosans was characterised by the grain of the population cultivar Piastowskie from the crop where the highest nitrogen fertilizer rate was applied ($140 \text{ kg N}\cdot\text{ha}^{-1}$). However, for the other rye cultivars, increasing nitrogen doses caused a decrease in the content of these compounds in the grain (Figure 5C). In 2020, as in 2019, the grain of the hybrid cultivars KWS Vinetto and SU Performer had the highest concentration of pentosans on the sites where no nitrogen fertilisation was applied, reaching 1.42 and 1.89% d.m., respectively. Population cultivars, on the other hand, responded with a significant increase in the content of water-soluble pentosans when the highest level of nitrogen fertilisation was applied, the content of these compounds being 1.67% d.m. (Figure 5D). Grabiński et al. [17] showed that higher nitrogen fertilisation increased water-soluble pentosans by 8.7%. A study by Noori [66] showed that both weather conditions, especially the amount of rainfall, as well as cultivar and nitrogen fertilisation, condition the pentosan content of wheat grain. Under drought conditions, despite higher fertilisation levels, the author obtained a lower pentosan content in grain than under higher nitrogen rates and optimum rainfall.

In 2019, grain of all rye cultivars tested showed the lowest trypsin inhibitor activity at the highest nitrogen fertilisation level applied in the experiment, of $140 \text{ kg N}\cdot\text{ha}^{-1}$ (Figure 5E). Grain of the hybrid cultivar KWS Vinetto had the highest trypsin inhibitor activity on the site without nitrogen fertilisation, as well as grain of the other cultivars after an application rate of $70 \text{ kg N}\cdot\text{ha}^{-1}$. In 2020, with the exception of the hybrid cultivar KWS Vinetto, the grain with the lowest trypsin inhibitor activity was obtained similarly to 2019 after a fertilisation application of $140 \text{ kg N}\cdot\text{ha}^{-1}$ (Figure 5F). In the case of the population cultivars Dańkowskie Skand and Piastowskie, the highest trypsin inhibitor activity was shown by the grain after an application rate of $70 \text{ kg N}\cdot\text{ha}^{-1}$ in the case of the hybrid cultivar KWS Vinetto $140 \text{ kg N}\cdot\text{ha}^{-1}$, and in the case of the hybrid cultivar SU Performer from a crop without nitrogen fertilisation. Studies on soybeans have shown that increasing rates of mineral nitrogen fertilisation result in a proportional decrease in trypsin inhibitor activity [67].

Xue et al. [68] found that dividing the nitrogen dose in relation to its single application resulted in a decrease in trypsin inhibitor activity in wheat grain. Grabiński et al. [17] showed that increasing the intensity of production technology, including nitrogen fertilisation, did not differentiate trypsin inhibitor activity in rye grain.

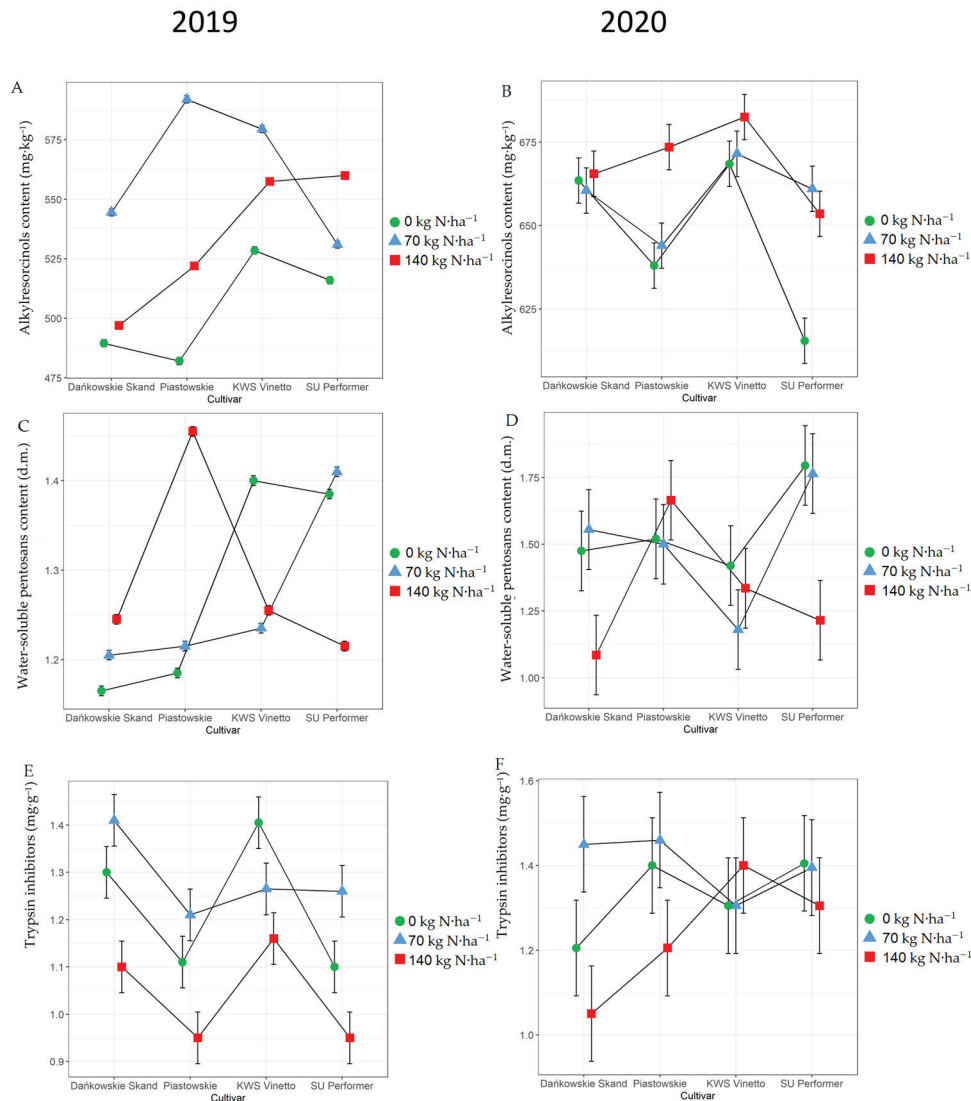


Figure 5. Interaction of nitrogen fertilisation and cultivar in the content of alkylresorcinols (A,B), water-soluble pentosans (C,D) and trypsin inhibitors (E,F) in rye grain.

Figure 6 shows the results of the PCA principal component analysis of the content of individual anti-nutrients in the grain of the rye cultivars tested in relation to the level of nitrogen fertilisation. We observe a strong positive correlation between yield and the content of alkylresorcinols in the grain. The hybrid cultivar KWS Vinetto showed the strongest correlation related to the application of a higher nitrogen dose and increased yield and alkylresorcinol content in grain. In contrast, the SU Performer hybrid variety showed the most-favourable response to fertilisation without significant changes in the content of water-soluble pentosans in the grain. We also observe a negative correlation between water-soluble pentosan content and trypsin inhibitor activity.

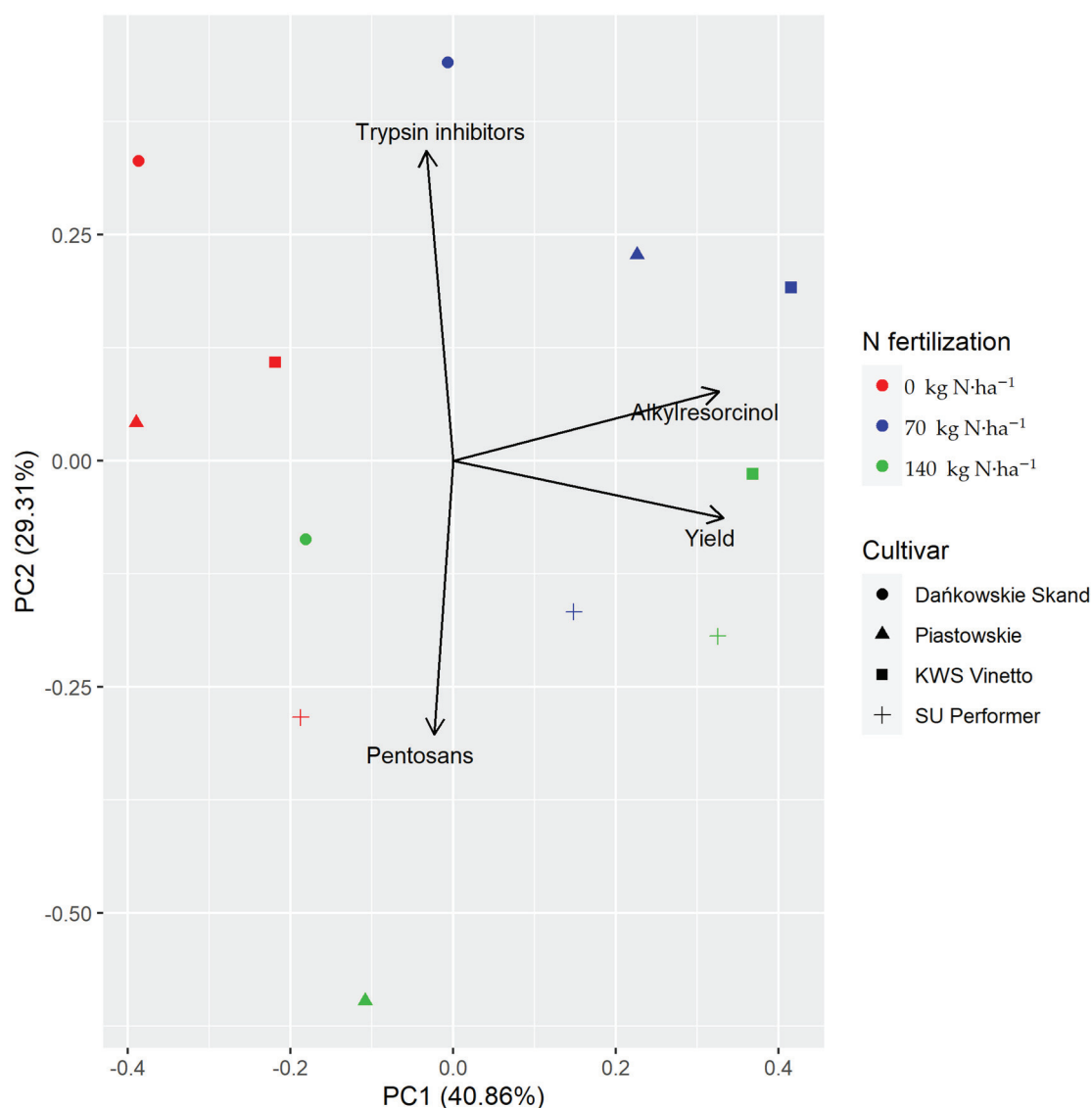


Figure 6. Biplot plot for PCA principal components for cultivar and nitrogen fertilisation as a function of alkylresorcinols, water-soluble pentosans and trypsin inhibitor activity and yield.

4. Conclusions

Our study showed that the contents of alkylresorcinols, water-soluble pentosans and trypsin inhibitors in rye grain depend on a cultivar. They are also affected by weather conditions and the level of nitrogen fertilisation. There was a correlation between cultivar and the rate of nitrogen fertilisation. A significantly greater synthesis of anti-nutritional substances in rye grain occurred in the growing season with high rainfall. The interaction of nitrogen fertilisation and cultivar affected the content of anti-nutritional substances in rye grain. The use of a higher nitrogen rate increased the content of alkylresorcinols to the largest degree at the KWS Vinetto hybrid cultivar. The Piastowskie population cultivar had the highest content of water-soluble pentosans in the grain at the highest nitrogen fertilisation rate used, while the Dańkowskie Skand population variety exhibited the highest activity of trypsin inhibitors in the grain at the low nitrogen fertilisation rate.

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Article

Abiotic and Biotic Factors Affecting Crop Growth and Productivity: Unique Buckwheat Production in Egypt

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Abstract: Egypt did not previously grow buckwheat, due to the belief that the environment does not meet the factors of growth, development, and productivity in an arid or semiarid region. The present study investigated two species of buckwheat, *Fagopyrum tataricum* (FT) and *Fagopyrum esculentum* (FE), which were planted in two different soil, weather, and water property sites, including the first in Belbies City and the second in Sadat City in the middle of January, November, and March for two successive seasons, 2018–2019 and 2019–2020. The study uniquely focuses on Egypt to investigate three interactions of location $\times$ species, location $\times$ sowing date, and species $\times$ sowing date on growth and productivity. The parameters measured included plant height (cm), number of branches, internodes, and leaves per plant, fresh weight (gm), number of grains per plant, grain weight of 1 m², and yield (kg/Ha). Our results indicated significant differences in all measured interactions. For the location $\times$ species interaction, FT planted in Belbies City consistently outperformed all other combinations, with a plant height = 97.704 cm in the 1st season and productivity = 859.38 kg/ha in the 1st season, while FE in Sadat showed the lowest growth and productivity. For the interaction of location $\times$ sowing date, Belbies $\times$ mid-March sowing achieved the highest plant heights of 84.89 cm and 75.44 cm, and productivity of 702.88 kg/ha and 708.21 kg/ha in consecutive seasons. Conversely, Sadat City $\times$ Mid-March sowing resulted in the lowest plant heights of 57.500 cm, and 57.667 cm, and productivity of 490.67 kg/ha, and 444.55 kg/ha. The species $\times$ sowing date interaction further emphasized the superiority of FT sown in mid-March, which led to the best plant height growth of 95.78 cm in the 1st season and the highest productivity of 837.55 kg/ha in the 1st season. In contrast, FE sown in mid-March exhibited the poorest outcomes. The study provides an understanding of the two-way interactions affecting buckwheat cultivation in Egypt. Our results indicated its viability with appropriate species selection and sowing dates, contributing to agricultural diversity and sustainability.

Keywords: buckwheat; Egyptian environment; *Fagopyrum tataricum*; *Fagopyrum esculentum*; interaction effect; productivity

1. Introduction

Agriculture, at the heart of global sustainability and food security, faces unprecedented challenges in the era of climate change [1]. Arid and semiarid regions, such as those predominant in Egypt, are particularly vulnerable, necessitating innovative agricultural practices and crop diversification. Among the various crops, buckwheat stands out for its exceptional adaptability, nutritional benefits, and potential to thrive under water-limited conditions, presenting an untapped resource for regions experiencing climatic adversity [2]. However, the exploration of underutilized crops such as buckwheat represents a frontier in agricultural research driven by the need to address food security and sustainable farming

practices in arid environments. Recent studies have begun to underscore the resilience of crops to poor soil conditions, drought, and low water availability, making them attractive options for enhancing agricultural diversity in challenging landscapes in Egypt [3,4]. The agricultural history of Egypt is marked by a reliance on water-intensive crops, an approach that is increasingly unsustainable given the country's water scarcity issues and the salinization of arable lands. The introduction of buckwheat, a crop previously overlooked due to misconceptions about its suitability, represents a paradigm shift toward sustainable agriculture, leveraging its minimal water requirement and adaptability [5]. However, this research challenges existing agricultural paradigms by demonstrating buckwheat's viability in Egypt, thereby contributing to the diversification of crops—a cornerstone of food security and ecological sustainability. By dissecting the interaction between abiotic and biotic factors, this study aimed to identify new agricultural practices that boost productivity while ensuring resilience against climate change [6,7]. Nevertheless, the interaction of genetic, environmental, and management factors is crucial in understanding crop growth and productivity, an area of agricultural science that has become increasingly relevant with the advent of climate change and the push for sustainable practices. Buckwheat has emerged as a crop of interest due to its adaptability to diverse climatic and soil conditions, underscoring the importance of this study within Egypt's unique agricultural patterns [8]. The variability in growth responses among buckwheat species to different environmental conditions highlights the significance of species selection, highlighting the complexity of genotype–environment interactions and their impact on crop performance across various settings [3,7,9,10]. Moreover, the influence of abiotic factors, such as soil conditions and external treatments, on buckwheat emphasizes the role of these factors in crop adaptability [6,11]. Environmental factors such as altitude and UV radiation also contribute to the complexity of buckwheat cultivation and have significant effects on growth and productivity [12,13]. Moreover, the impact of sowing date on buckwheat yield illustrates the significance of temporal factors in agricultural outcomes [5,14,15]. Challenges posed by seasonal transitions and specific soil conditions, such as salinity, necessitate a deeper understanding of optimal cultivation practices [4,16]. However, soil health and management practices play a critical role in determining crop productivity, highlighting the importance of soil conditions and agronomic practices in achieving sustainable yields [17–19]. The history of land use and previous cropping systems influences the success of current crops, revealing the intricate relationship between past and present agricultural practices [20,21].

Furthermore, the classification of *Fagopyrum* sp. involves distinguishing between *F. esculentum* and *F. tataricum*, which are species whose biological differences are emphasized. *F. esculentum*, commonly known as a common buckwheat species, is an annual Asian herb with small pinkish or white flowers and edible triangular seeds. In contrast, *F. tataricum*, which is also an erect annual herb, has a smaller seed size. Studies have shown that *F. esculentum* flowers earlier than *F. tataricum* and produces fewer nodes, branches, and inflorescences but more flowers per inflorescence [2,17]. Additionally, *F. tataricum* is more resistant to water stress and exhibits traits of drought tolerance, while *F. esculentum* has characteristics of drought avoidance [22]. Furthermore, there are differences in genome size between these species, with *F. esculentum* having a larger genome size than *F. tataricum* [7,18]. However, the influence of location, species choice, and sowing date on buckwheat growth and productivity, as well as the variability in certain buckwheat species in response to environmental conditions [3,9] and the role of genotype–environment interactions [7,10], and the effects of external treatments and soil conditions on buckwheat plants further expand our understanding of these interactions [6,11]. Additionally, the influence of altitude and UV radiation provides further context for understanding the environmental sensitivity of buckwheat [12,13], further to the impact of sowing date on buckwheat productivity [5,14,15]. Nevertheless, the transition between seasons [16] and the specific challenges of saline soils [4] further contribute to the understanding of the optimal conditions for buckwheat cultivation and the critical role of soil conditions in crop

productivity. Previous crops and cultivation practices were influenced, along with insights into weed suppression and no-tillage systems [17–21,23–25].

This research investigated the interaction between biotic and abiotic factors in buckwheat cultivation in arid regions of Egypt, aiming to optimize growth conditions and unlock the potential for resilience and nutrition in sustainable farming systems. By exploring buckwheat's viability in these challenging climates, this study challenges traditional agricultural practices and offers insights into enhancing food security and sustainability amid environmental challenges. These findings suggest that selecting appropriate species and sowing dates, in addition to suitable farming practices, can significantly improve buckwheat productivity in Egypt, supporting the goals of agricultural diversification and sustainability in response to changing climate conditions.

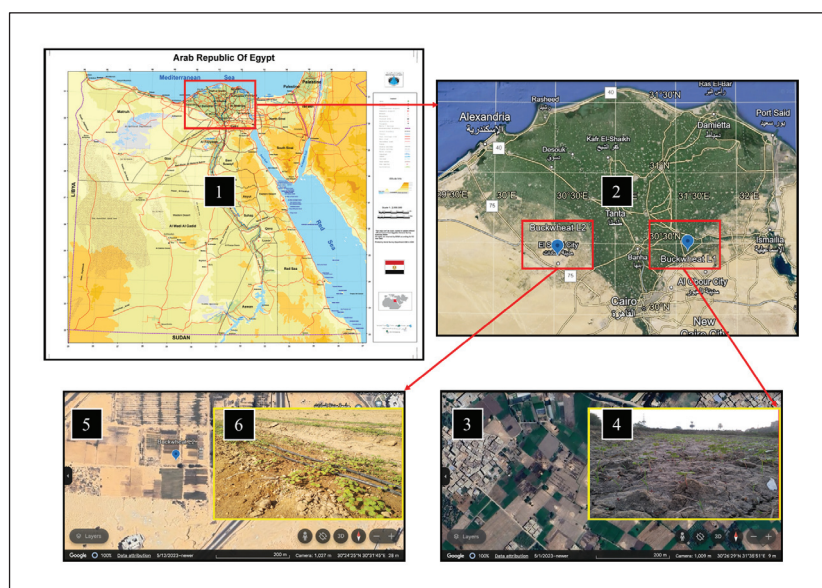
2. Materials and Methods

Field experiments were conducted during the 2018–2019 and 2019–2020 seasons at two locations: the Belbies City site (BCS) and the experimental farm of the Environmental Studies & Research Institute (ESRI), University of Sadat City (SCS) in Sadat City, Monofiya. These experiments aimed to evaluate the growth and productivity of buckwheat plants under varying climatic conditions, focusing on the morphological characteristics and yield of grains from different buckwheat species and sowing dates.

The experimental treatments were as follows:

2.1. Materials

Locations of Cultivation: BCS Was Located at 30.4196° N, 31.5619° E, and SCS Was Located at 30.3594° N, 30.5327° E (Scheme 1).



Scheme 1. The study locations where the topography of each planting site showed, where: 1 = Map of Egypt (sourced by CAMPASS, 2023). 2 = A focused satellite image of the regions of both study locations (by Google Earth Web, 2023). 3 & 4 are satellite images of the planting sites of Belbies City and Sadat City, respectively (sourced by Google Earth Web, 2023). 5 & 6 are real images of the planting sites at the early stages of growth in Belbies City and Sadat City, respectively. Photos edited and compiled by the authors.

Species: *Fagopyrum esculentum*, Represented by “Japanese”; *Fagopyrum tataricum*, by “Madawaska”; Both Cultivars were Purchased Online from a Commercial Seed Company in California, United States, and Shipped to Egypt following the force Laws of the State Ministry of Agriculture for Quarantining and Importing Seeds.

Sowing Dates

Season 1: Second week of November 2018, January 2019, and March 2019

Season 2: Second weeks of November 2019, January 2020, and March 2020

2.2. Methods

2.2.1. Seeds

The seeds were sealed in their original bags and then opened in the field for planting. The first batch was planted at the Belbies City site as afeer cultivation [14] (dry seeds in a dry land, a common planting method widespread in the cultivation of grain, e.g., wheat). The seeds were mixed with sand at a 1:2 *v/v* ratio to control the spread of the seeds on the designated plot and ensure that all the seeds were well sown with uniformity. The experiment used a rate of 160 seeds/m² for all 3 sowing dates at both locations, and the first planting occurred at the Belbies City site in mid-November 2018. A total of 6 plots were planted in each patch. Three plots were randomly distributed for *F. esculentum*, and three were allocated for *F. tataricum*; each plot was an area of 4.5 m² planted with ± 720 seeds weighing approximately 21.800 g. In the second location, the seeds were sowed manually by clever farmers and then land scarfed via a handy fork to ensure the uniformity of the seeds and ensure that the soil was covered by a layer of soil at least 2 cm in length. However, due to the land structure, a raised bed is commonly used there, so the seeds were spread on the upper side of the raised bed where each plot was 6 m $\times$ 0.75 m = 4.5 m², with the same rate of seed and sand mixture used in the first location. Irrigation was applied to both sites directly after sowing. However, at the location of Sadat City, the raised beds were covered by plastic sheets to avoid bird attacks.

2.2.2. Experimental Site Analysis

At the beginning of each experimental season, three soil samples from the top layer (0–30 cm) were randomly collected from the experimental sites. Then, the samples were air dried, ground, and sieved through a 2 mm sieve and then bulked together before being subjected to soil analysis for classifying soil texture through particle size distribution analysis (mechanical analysis) as well as determining some soil chemical characteristics [7]. The physical and chemical properties of the experimental soil during both seasons of the study are presented in Table 1, and the water properties, including physical, chemical, and biological parameters, were analysed at both sites, as shown in Table 2. However, weather data for both study sites, including temperature, total precipitation, relative humidity, wind gusts, sunshine duration, and soil temperature [0–7 cm down], were obtained by the weather model Nonhydrostatic Meso-Scale Modelling Technology (NMM) of Meteoblue based in Basil, Switzerland, as shown in Table 3.

Table 1. Physical and chemical properties of the experimental soil samples.

Location	Parameter	Belbies City Site	Sadat City Site
Soil Properties			
pH	-	7.83	8.57
EC (dS/m)	Electrical Conductivity	1.48	1.11
SP %	Saturation Percentage	25	-
Soluble Anions (meq/L)			
CO ₃ ²⁻	Carbonate	-	-
HCO ₃ ⁻	Bicarbonate	0.25	1.9
Cl ⁻	Chloride	12.1	7.4
SO ₄ ²⁻	Sulfate	1.56	3.4

Table 1. *Cont.*

Location	Parameter	Belbies City Site	Sadat City Site
Soluble Cations (meq/L)			
Ca ²⁺	Calcium	3.7	1.2
Mg ²⁺	Magnesium	1.8	1.2
Na ⁺	Sodium	8	3.3
K ⁺	Potassium	0.5	0.18
Soil Composition			
CaCO ₃ %	Calcium Carbonate	3.9	1.7
OM %	Organic Matter	1.2	0.35
Available Nutrients (mg/kg)			
N	Nitrogen	175	-
P	Phosphorus	183.6	5.3
K	Potassium	7.96	508
Cu	Copper	0.75	1.43
Fe	Iron	5.25	1.11
Mn	Manganese	2.43	20
Zn	Zinc	2	2.75
Mechanical Analysis (%)			
Sand	-	83	83
Silt	-	15.5	10
Clay	-	1.2	7
Texture Grade	-	Clay sandy	Sandy loamy

Notes: EC (dS/m): Reflects soil salinity; lower values indicate less salinity. OM %: Indicates soil fertility; higher values suggest more organic matter and better soil health. Soluble anions and cations are expressed in milliequivalents per liter (meq/L), indicating the concentration of each ion in the soil solution. Available levels of nutrients (P, K, Cu, Fe, Mn, Zn) are now correctly presented in milligrams per kilogram (mg/kg) for clarity, reflecting the concentration of each nutrient available in the soil for plant uptake. The “-” indicates data not available or not applicable.

Table 2. Irrigation water properties of the experimental soil samples.

Parameter	Unit	Belbies City Site	Sadat City Site
pH	-	7.41	7.98
EC	dS/m	0.64	1.26
Chemical Parameters			
Ca ²⁺	meq/L	1.70	4.20
Mg ²⁺	meq/L	1.00	2.60
Na ⁺	meq/L	3.20	6.50
K ⁺	meq/L	0.01	0.20
CO ₃ ²⁻	meq/L	0.00	0.00
HCO ₃ ⁻	meq/L	0.21	4.00
Cl ⁻	meq/L	4.70	7.00
SO ₄ ²⁻	meq/L	10.9	2.50
SAR	-	2.76	3.50
TDS	mg/L	410.00	896.00
Trace Elements			
Fe	ppm	0.27	0.11
Zn	ppm	0.01	0.18
Mn	ppm	0.20	<0.01
Cu	ppm	0.02	<0.01

Notes: EC (Electrical Conductivity) is measured in deciSiemens per meter (dS/m), indicating the water’s salinity level. Lower values suggest lower salinity, which is generally better for irrigation purposes. SAR (Sodium Adsorption Ratio) is a measure of the suitability of water for use in agricultural irrigation, based on the sodium (Na⁺) content relative to calcium (Ca²⁺) and magnesium (Mg²⁺) contents. TDS (Total Dissolved Solids) is measured in milligrams per liter (mg/L), reflecting the total concentration of dissolved substances in water. Higher TDS values indicate more dissolved minerals and salts. Trace elements like Fe (Iron), Zn (Zinc), Mn (Manganese), and Cu (Copper) are presented in parts per million (ppm).

Table 3. Weather data of the study locations.

Parameter \ Location	Belbies City Site (BCS)		Sadat City Site (SCS)	
	Season 1 (2018–2019)	Season 2 (2019–2020)	Season 1 (2018–2019)	Season 2 (2019–2020)
Temperature [°C] Max	27.31–36.71	19.04–35.8	18.37–35.86	17.9–33.92
Temperature [°C] Min	8.51–24.48	9.75–21.27	7.97–23.87	9.57–20.64
Temperature [°C] Mean	13.65–30.37	14.18–28.24	12.92–29.33	13.61–26.81
Precipitation [mm] Total	0.00–0.47	0.00–1.42	0.00–2.00	0.00–1.12
Relative Humidity [%] Max	68.3–85.94	75.52–81.57	70.88–89.44	79.23–84.67
Relative Humidity [%] Min	19.63–33.3	22.22–35.56	21.26–37.89	24.26–42.92
Wind Gust [km/h] Max	27.53–40.62	30.6–39.4	28.36–41.82	30.7–41.12
Sun Duration [min] Total	492.23–777.84	413.8–804.67	514.65–788.43	402.65–802.5
Soil Temperature [°C] Max	28.01–40.08	19.7–38.58	20.32–43.27	20.31–40.71
Soil Temperature [°C] Min	11.27–28.89	12.57–26.29	9.00–26.96	10.87–23.56
Soil Temperature [°C] Mean	15.22–34.03	15.09–31.85	13.98–33.97	15.02–31.14

2.2.3. Experimental Site Preparation

The experimental soil was prepared by land plowing in two perpendicular directions, and all the experimental plots received organic manure as compost at a rate of 16 tons/hectare, phosphorus fertilizer at 80 units P_2O_5 /hectare as calcium superphosphate (15.5% P_2O_5), and agricultural sulfur as agricultural sorghum. Superfine 98% S at 180 kg/hectare full doses of compost, superphosphate, and agricultural sulfur were applied once during the final preparation of the experimental soil and thoroughly mixed with the soil before the ridges were constructed. However, nitrogen fertilizer was applied at 180 units N/hectare in the form of ammonium sulfate (20.6% N). The nitrogen dose was split into 3 portions. The first portion was added 15 days after the seeding date, and the remaining portions were subsequently added at a rate of one portion every 15 days until grain setting. Nitrogen fertilizer was applied manually at the Belbies site and through injection in a drip irrigation system as a plant fertigation application at the Sadat site.

2.2.4. Data Recorded

Plant Vegetative Growth Parameters

After 70 days after sowing and after all the experimental treatments were carried out, a random and representative sample of 9 buckwheat plants from each experimental subplot was taken by cutting at ground level (at 2–3 cm above the ground surface) and subsequently transferred to the laboratory of the Environmental Studies and Research Institute to determine the following vegetative growth parameters.

Plant Height (cm)

The average plant height was recorded in centimeters (cm) from the point of contact of the plant stem with the soil up to the highest point of the inflorescence on the main stem of the buckwheat plant samples in each experimental subplot.

Number of Branches per Plant

The average number of lateral branches per plant was counted for the buckwheat plant samples in each experimental subplot, and the average number of branches per plant was calculated.

Whole-Plant Fresh Weight (gm)

The average fresh weight of the whole buckwheat plant (stem, lateral branches, and leaves) was recorded in grams (gm) by using a 3-decimal digital electronic balance directly after being taken from the fields of the study.

Harvesting of Buckwheat Grain Samples

At the harvesting stage, ± 75 days after the sowing date, a representative sample of 9 individual buckwheat plants (group A plants), in addition to the plant samples harvested from one square meter from the middle of each experimental subplot (group B plants), was randomly taken by cutting at ground level (at 2–3 cm above the ground surface). Then, all the plant samples were left to air dry for at least 7–10 days for the plants planted in mid-November and January and for 3–5 days for the plants planted in mid-March. Afterward, each of the 10 dried plant samples was threshed by hand to obtain the grains from the dried main and lateral panicles per group of plants (group A plants) or per all plant samples harvested from each experimental subplot (group B plants). Moreover, the buckwheat grains were manually ground to clean and remove any impurities, foreign matter, or broken or immature grains. The cleaned grains of group B plants were used directly to determine the number of grains/plant, total grain yield per square meter (kg), and grain yield (kg/Ha), where Ha = 10,000 square meters.

Number of Seeds per Plant

The average number of seeds per plant in each experimental subplot was determined by manually counting the grains extracted from 9 plants in the dried main and lateral panicles of individual plants in group A. The total number of seeds was subsequently divided by 9 to obtain the grain count per plant.

Total Grain Weight per Square Meter (kg)

The average total grain yield per square meter was calculated by quantifying the number of harvested plants (group B) within one square meter. Then, the dried plants were threshed by hand to obtain the grains from the dried main and lateral panicles, and a 3-decimal-digit electronic balance was used to obtain the average grain yield/meter² in kilograms (kg) units.

Total Grain Yield per Hectare (kg/ha)

The average total grain yield per hectare was calculated by multiplying the average grain yield/square meter (g) by the average number of buckwheat plants per hectare (a plant density of approximately 122 thousand plants per hectare) to obtain the average grain yield/hectare in kilograms per hectare (kg/ha).

2.3. The Statistical Analysis

A randomized complete block design incorporating three factors was used for the data analysis, with each parameter being replicated thrice. The treatment means were subjected to pairwise comparison using the least significant difference (LSD) test, as described by

Snedecor and Cochran (1994) [26]. The data analysis was performed utilizing the Assistat software program, version 1.0.

3. Results

3.1. Evaluation of the 2-Way Interaction Effect of Biotic and Abiotic Factors on the Growth of Buckwheat

3.1.1. Interaction between Location and Species

Through our results shown in Table 4 and Figure 1, we observed interaction effects between location (Belbies City vs. Sadat City) and species (*Fagopyrum tataricum* vs. *Fagopyrum esculentum*) on various growth characteristics of buckwheat over two seasons. For plant height, in both seasons, *Fagopyrum tataricum* plants grown in Belbies City had the highest average plant height, significantly surpassing those of all the other combinations. The least significant difference (LSD) at the 0.05 level highlights the statistical significance of these differences, clearly indicating the superior growth performance of *Fagopyrum tataricum* in Belbies City. For the branch number per plant, similar patterns were observed for the number of branches, with *Fagopyrum tataricum* in Belbies City producing the most branches compared to the other groups. The differences between the groups were statistically significant, as confirmed by the L.S.D., underscoring the combined influence of species and location on this growth characteristic. For the internode number per plant, the trend continues with the number of internodes, where *Fagopyrum tataricum* in Belbies City exhibited the highest count, significantly outperforming the buckwheat grown under other conditions. These results are consistent across both seasons, with the L.S.D. values affirming the significance of these findings. For the number of leaves per plant, *Fagopyrum tataricum* in Belbies City also had a significantly greater number of leaves than the other plants, indicating that a robust growth pattern was influenced by both the choice of species and the cultivation location. These differences were validated by the corresponding L.S.D. values. Finally, for the fresh weight per plant, to reflect the trends in the aforementioned parameters, *Fagopyrum tataricum* plants grown in Belbies City also had the highest fresh weight per plant, indicating that this combination was the most productive in terms of biomass accumulation. The statistical analysis, supported by the L.S.D. values, confirmed the impact of species and location on this critical growth metric.

Table 4. Interaction between location and species and its effect on buckwheat growth characteristics.

Parameters	Treatments	<i>Fagopyrum tataricum</i>		<i>Fagopyrum esculentum</i>		L.S.D. at 0.05
		Belbies City	Sadat City	Belbies City	Sadat City	
Plant height cm per plant	1st Season	97.704 a	85.667 b	75.296 c	35.463 d	6.6183
	2nd Season	90.741 a	78.333 b	63.185 c	41.704 d	3.6779
Branch number per plant	1st Season	12.000 a	10.111 b	8.000 c	6.185 d	0.476
	2nd Season	10.704 a	9.445 b	7.778 c	6.815 d	0.334
Internode number per plant	1st Season	14.111 a	11.037 b	8.889 c	7.185 d	0.597
	2nd Season	12.259 a	10.889 b	9.037 c	7.296 d	0.3517
Number of leaves per plant	1st Season	46.852 a	27.889 b	18.778 c	11.037 d	2.6427
	2nd Season	31.778 a	19.630 b	14.370 c	8.556 d	2.872
Fresh weight per plant gm	1st Season	36.188 a	22.187 b	14.330 c	10.171 d	1.6143
	2nd Season	39.356 a	24.771 b	16.013 c	11.31 d	1.881

where L.S.D. = least significant difference. Different letters within the same row indicate significant differences ($p \leq 0.05$).

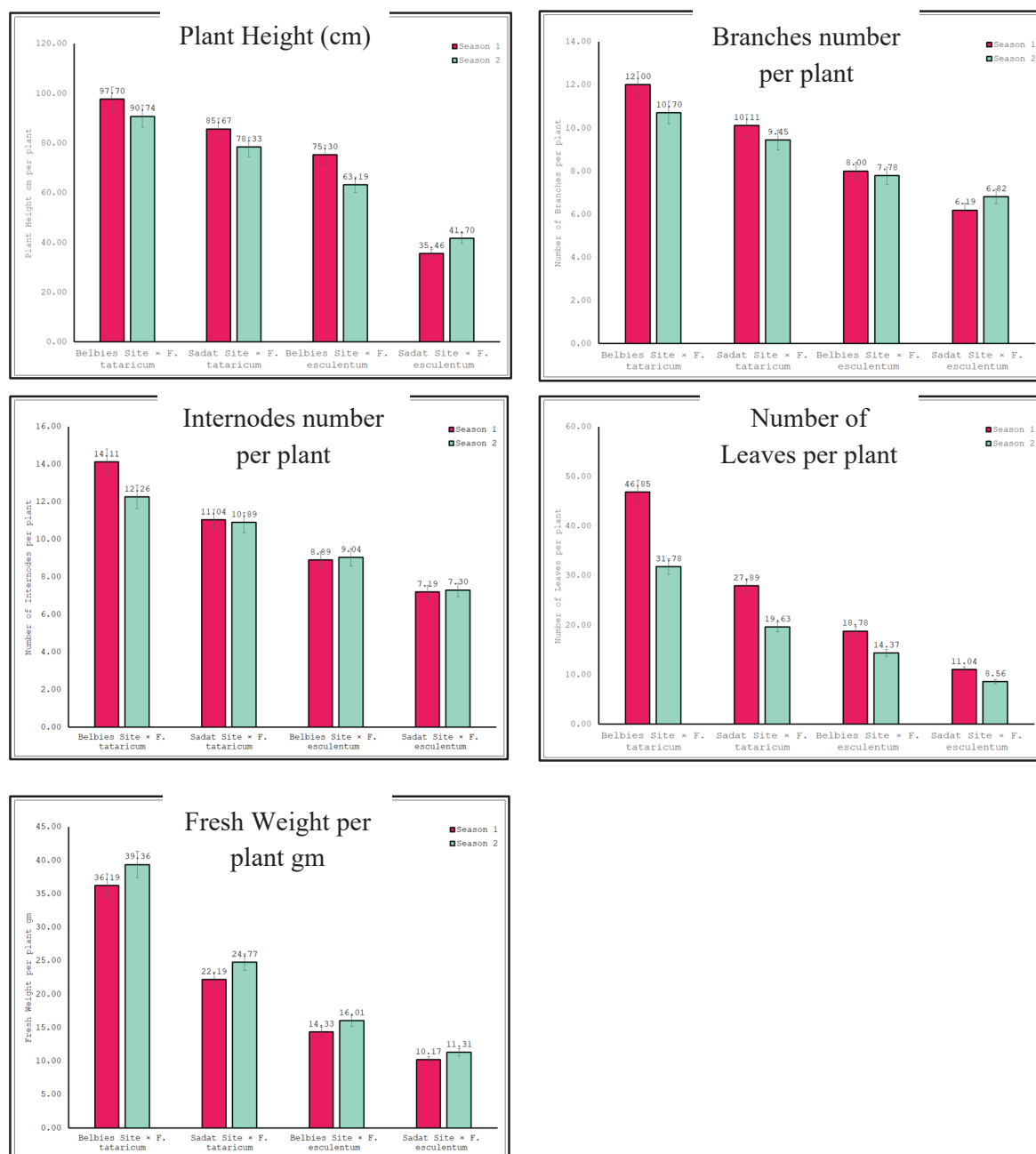


Figure 1. Evaluation of location × species on the growth of buckwheat.

3.1.2. Interaction of Location × Planting Date

The results in Table 5 and Figure 2 show the interaction effects of location and sowing time on the growth of buckwheat plants across two parameters: physical growth characteristics and fresh weight per plant. The study differentiates results by location (Belbies City site vs. Sadat City site) and by sowing time (mid-January, mid-November, mid-March), presenting findings for two growing seasons. For plant height, in both the first and second seasons, plant height was consistently greater in Belbies City than in Sadat City across all sowing times. The least significant difference (L.S.D.) at the 0.05 level for plant height indicates significant differences between locations, with no significant variation among sowing times within the same location. For instance, in the first season, plant heights in Belbies City ranged from 84.889 cm to 88.222 cm, which was significantly greater than those in Sadat City, where heights ranged from 57.500 cm to 62.806 cm ($p \leq 0.05$). For the branch and internode number, the number of branches and internodes per plant followed a similar

pattern to that of plant height, with plants in Belbies City exhibiting more branches and internodes than those in Sadat City, regardless of sowing time. The L.S.D. values confirmed these differences as statistically significant ($p \leq 0.05$), demonstrating the impact of location over sowing time on these growth parameters. The number of leaves per plant showed notable variability, with significant differences observed not only between locations but also among sowing times within the same location, especially in the first season. For example, in Belbies City, during the first season, sowing in mid-March resulted in the highest number of leaves (35.778), which was significantly greater than that of the other sowing times, indicating that both location and sowing time influence leaf production. In terms of fresh weight per plant, fresh weight per plant demonstrated significant variation influenced by both location and sowing time, with higher weights generally observed in Belbies City across all sowing times. In the second season, sowing in mid-March in Belbies City resulted in the highest fresh weight (28.857 g), which was significantly greater than that of all the measurements in Sadat City, where the maximum observed weight was 20.921 g for mid-March sowing.

Table 5. Interaction effect of location $\times$ sowing time on the growth of buckwheat plants.

Parameters	Treatments	Belbies City Site			Sadat City Site			L.S.D. at 0.05
		Mid-Jan	Mid-Nov	Mid-Mar	Mid-Jan	Mid-Nov	Mid-Mar	
Plant height cm per plant	1st Season	88.222 a	86.389 a	84.889 a	62.806 b	61.389 b	57.500 b	8.106
	2nd Season	77.667 a	77.778 a	75.444 a	60.444 b	61.945 b	57.667 b	4.504
Branch number per plant	1st Season	10.056 a	9.944 a	10.000 a	8.222 b	8.111 b	8.111 b	0.583
	2nd Season	9.223 a	9.111 a	9.389 a	8.111 b	8.222 b	8.056 b	0.409
Internode number per plant	1st Season	11.611 a	11.444 a	11.445 a	9.000 b	9.056 b	9.278 b	0.731
	2nd Season	10.778 a	10.778 a	10.389 a	9.222 b	9.222 b	8.833 b	0.431
Number of leaves per plant	1st Season	32.500 a	30.167 b	35.778 b	17.722 c	19.444 cd	21.222 d	3.237
	2nd Season	21.889 b	21.889 b	25.444 a	13.834 c	14.500 c	13.944 c	3.517
Fresh weight per plant gm	1st Season	24.817 a	24.323 ab	26.637 b	15.433 c	15.239 d	17.810 d	1.977
	2nd Season	26.591	27.604 a	28.857 a	16.222 c	16.979 c	20.921 b	2.304

where L.S.D. = least significant difference. Different letters within the same row indicate significant differences ($p \leq 0.05$).

3.1.3. Interaction Effect of Species $\times$ Sowing Time

Our results in Table 6 and Figure 3 detail the interaction effects of species (*Fagopyrum tataricum* $\times$ *Fagopyrum esculentum*) and planting time (mid-March, mid-January, mid-November) on the growth of buckwheat across various growth parameters over two growing seasons. For plant height, the data showed that, compared with *Fagopyrum esculentum*, *Fagopyrum tataricum* consistently achieved greater plant heights across all planting times, with significant differences noted ($p \leq 0.05$). For instance, in the first season, the height reached 95.778 cm for *Fagopyrum tataricum* sown in mid-March, which was significantly taller than that of *Fagopyrum esculentum* in the same period, which reached only 46.611 cm. An L.S.D. of 0.05 validates the statistical significance of these differences. In terms of the number of branches, similar trends were observed for the number of branches, where *Fagopyrum tataricum* plants consistently had more branches than *Fagopyrum esculentum* plants across all planting times. The first season showed a significant difference with *Fagopyrum tataricum* sown in Mid-March having 11.722 branches, contrasting with 6.389 branches for *Fagopyrum esculentum* sown in the same period, supported by a statistically significant L.S.D. The number of internodes followed a pattern akin to plant height and branch number, with *Fagopyrum tataricum* exhibiting a higher number of internodes than *Fagopyrum esculentum*. This effect was consistent across all planting times and both seasons, demonstrating the influence of the species on this growth parameter. However, for the number of leaves, *Fagopyrum tataricum* plants also had a significantly greater number of leaves than *Fagopyrum esculentum* plants, with the greatest disparity observed in the first season. The difference in leaf count between the species was statistically significant,

as indicated by the L.S.D. values. Finally, for fresh weight per plant, the fresh weight of *Fagopyrum tataricum* plants was significantly greater than that of *Fagopyrum esculentum* plants across all planting times and seasons. This parameter clearly illustrates the superior growth performance of *Fagopyrum tataricum* under the conditions tested.

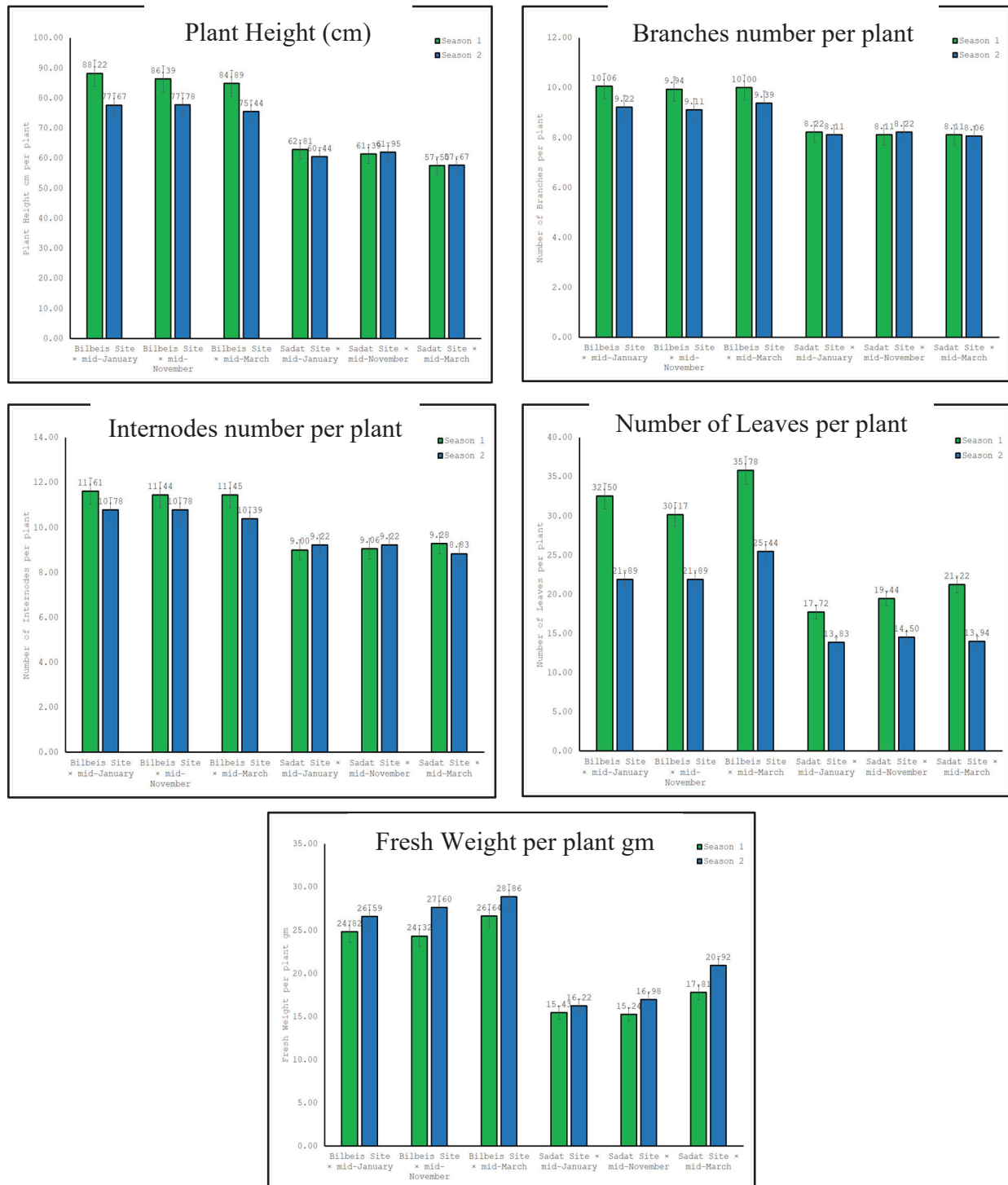
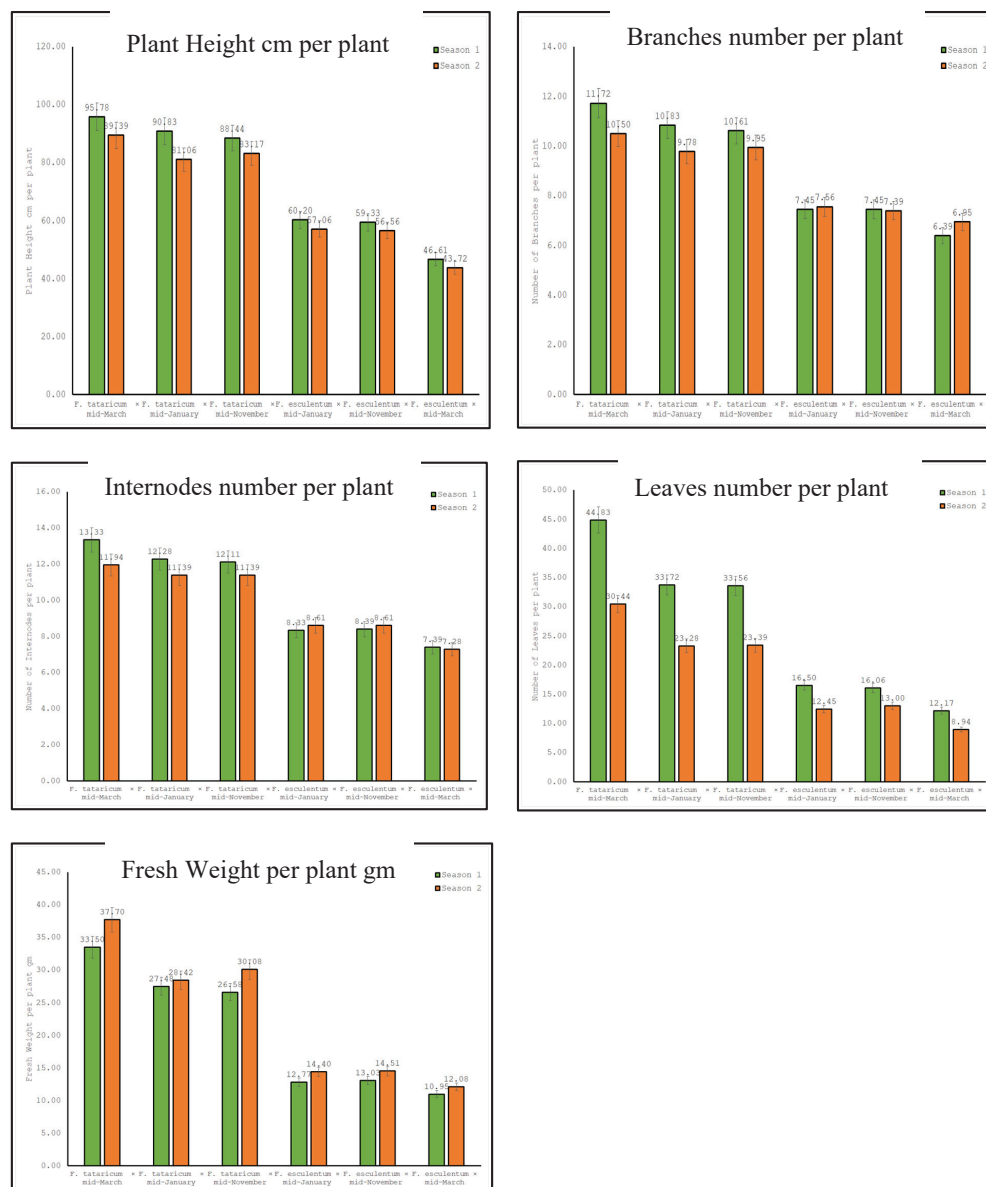


Figure 2. Evaluation of location × sowing date on the growth of buckwheat.

Table 6. Interaction effect of species $\times$ planting times on the growth of buckwheat.

Parameters	Treatments	<i>Fagopyrum tataricum</i>			<i>Fagopyrum esculentum</i>			L.S.D. at 0.05
		Mid-Mar	Mid-Jan	Mid-Nov	Mid-Jan	Mid-Nov	Mid-Mar	
Plant height cm per plant	1st Season	95.778 a	90.833 a	88.444 a	60.195 b	59.333 b	46.611 b	8.1057
	2nd Season	89.389 a	81.056 b	83.167 b	57.056 c	56.556 c	43.722 d	4.5045
Branch number per plant	1st Season	11.722 a	10.833 b	10.611 b	7.445 c	7.445 c	6.389 d	0.583
	2nd Season	10.500 a	9.778 b	9.945 b	7.556 c	7.389 c	6.945 d	0.4091
Internode number per plant	1st Season	13.334 a	12.278 b	12.111 b	8.334 c	8.389 c	7.389 d	0.7312
	2nd Season	11.944 a	11.389 b	11.389 b	8.611 c	8.611 c	7.278 d	0.4307
Leaf number per plant	1st Season	44.833 a	33.722 b	33.556 b	16.500 c	16.055 c	12.166 d	3.3267
	2nd Season	30.444 a	23.278 b	23.389 b	12.445 c	13.000 cd	8.944 d	3.5174
Fresh weight per plant gm	1st Season	33.495 a	27.483 b	26.584 b	12.767 c	13.032 cd	10.952 d	1.977
	2nd Season	37.697 a	28.418 b	30.075 b	14.395 c	14.508 c	12.081 d	2.3037

where L.S.D. = least significant difference. Different letters within the same row indicate significant differences ($p \leq 0.05$).

**Figure 3.** Evaluation of species $\times$ sowing date on the growth of buckwheat.

3.2. Evaluation of the 2-Way Interaction between Biotic and Abiotic Factors on the Productivity of Buckwheat

3.2.1. Interaction between Location and Species

The obtained results in Table 7 and Figure 4 show the interaction effects of location (belbies vs. Sadat) and species (*Fagopyrum tataricum* vs. *Fagopyrum esculentum*) on the productivity of buckwheat plants, examining parameters such as the number of seeds per plant, grains per square meter, and productivity rate per hectare over two growing seasons. For the number of seeds per plant, *Fagopyrum tataricum* in Belbies City exhibited the highest number of seeds per plant in both seasons (48.07 and 49.63, respectively), significantly outperforming all the other treatment combinations. *Fagopyrum esculentum* in Sadat City had the lowest seed count (13.67 and 14.63, respectively). A least significant difference (LSD) of 0.05 was used to confirm these differences as statistically significant. Similarly, *Fagopyrum tataricum* grown in Belbies City achieved the highest grain yield per square meter (0.0886 kg and 0.0867 kg, respectively, in the first and second seasons), markedly higher than the lowest yields observed for *Fagopyrum esculentum* in Sadat City (0.0314 kg and 0.032 kg, respectively). The LSD test results validated the significant impact of both location and species on grain yield. Additionally, for the kg/Hectare productivity rate, which reflects trends in the other parameters, *Fagopyrum tataricum* in Belbies City also led in terms of productivity per hectare, with 859.38 kg and 832.27 kg in the first and second seasons, respectively. In contrast, *Fagopyrum esculentum* in Sadat City had the lowest productivity (316.32 kg and 319.12 kg, respectively). The statistical significance of these differences was confirmed by the LSD test at 0.05.

Table 7. Interaction effect of location $\times$ species on the productivity of buckwheat.

Treatment	Parameter	Number of Seeds Per Plant		Grains of 1 Meter ² (kg)		Productivity Rate kg/Hectare	
		1st Season	2nd Season	1st Season	2nd Season	1st Season	2nd Season
<i>Fagopyrum tataricum</i>	Belbies	48.07 a	49.63 a	0.088.6 a	0.0867 a	859.38 a	832.27 a
	Sadat	25.04 b	26.74 b	0.0663 b	0.0596 b	661.43 b	594.43 b
<i>Fagopyrum esculentum</i>	Belbies	17.74 c	18.59 c	0.0499 c	0.0467 c	498.02 c	467.72 c
	Sadat	13.67 d	14.63 d	0.0314 d	0.032 d	316.32 d	319.12 d
LSD at 0.05		3.407	3.1064	3.85×10^{-3}	5.82×10^{-3}	39.714	60.144

where L.S.D. = least significant difference. Different letters within the same row indicate significant differences ($p \leq 0.05$).

These results delineate a clear pattern of superior performance by *Fagopyrum tataricum*, especially when cultivated in Belbies City, across all the measured productivity parameters. Conversely, *Fagopyrum esculentum* in Sadat City consistently exhibited the lowest productivity. The statistically significant differences, as indicated by the LSD tests, affirm that both the choice of species and the cultivation location critically influence buckwheat productivity.

3.2.2. Interaction of Location $\times$ Sowing Time

Our findings in Table 8 and Figure 5 explore the interaction effect of location (Belbies City site vs. Sadat City site) and sowing time (mid-January, mid-November, and mid-March) on the productivity of buckwheat, focusing on the number of seeds per plant, grains per square meter, and the productivity rate per hectare across two growing seasons. For the number of seeds per plant, the data clearly illustrate the influence of both sowing time and location on the number of seeds per plant. In both seasons, sowing at the Belbies City site in mid-March resulted in the highest number of seeds (37.72 and 37.83, respectively), significantly surpassing the counts in Sadat city at any sowing time. A least significant difference (LSD) of 0.05 was used to confirm the statistical significance of these observations. Similarly, the highest grain yield per square meter was observed for buckwheat sown in mid-March at the Belbies City site (0.0723 kg and 0.073 kg in the first and second seasons, respectively), indicating optimal productivity when sown during this period. Again, the

yields were significantly lower in Sadat City, regardless of the sowing time, with the LSD values substantiating the significance of the interaction between location and sowing time. However, for the kg/Hectare productivity rate, which reflects the trends in number of seeds per plant and grains per square meter, the highest productivity rates per hectare were achieved with mid-March sowing at the Belbies City site (702.88 kg and 708.21 kg in the first and second seasons, respectively). In contrast, all the productivity metrics in Sadat City were significantly lower, underscoring the influence of both sowing date and location on overall productivity. The differences across these groups were statistically significant, as indicated by the LSD at 0.05.

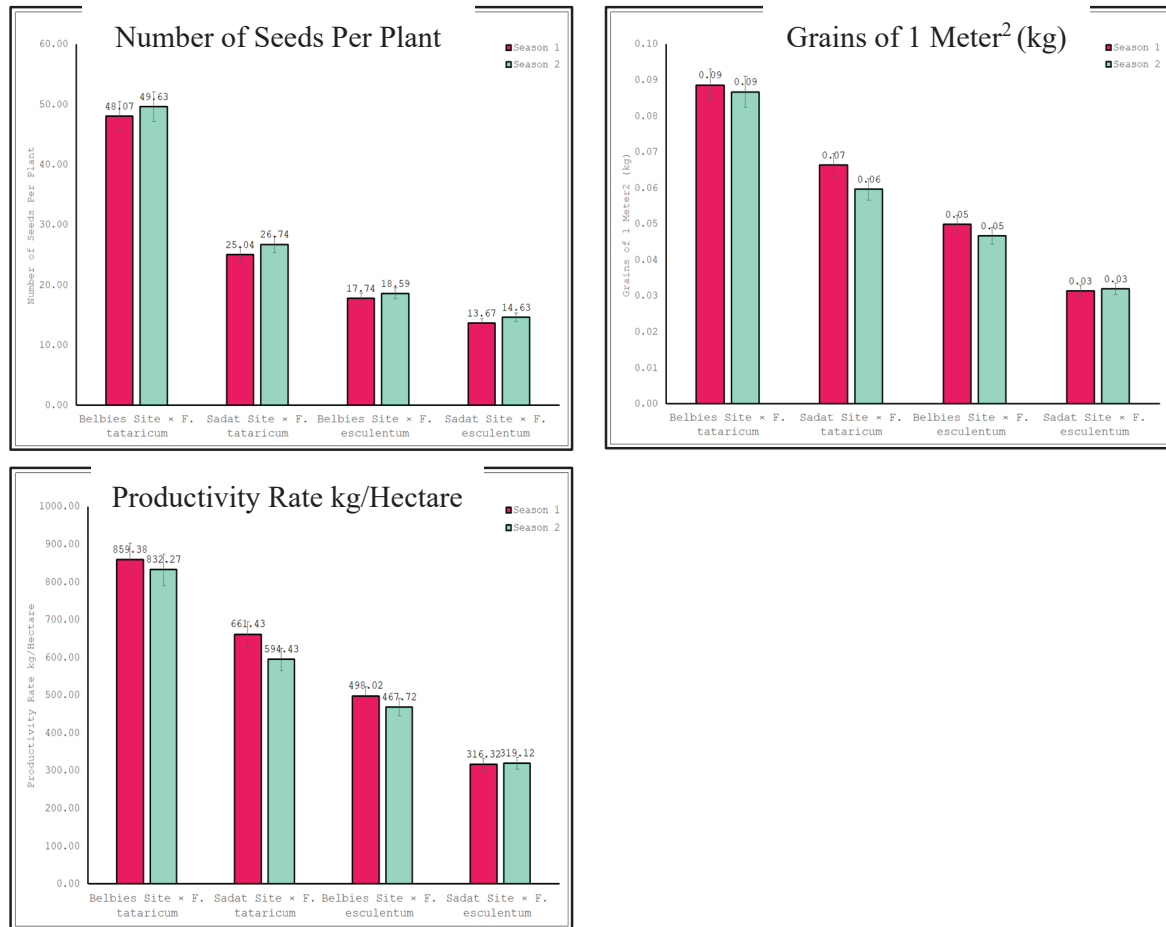


Figure 4. Evaluation of location × species on the productivity of buckwheat.

Table 8. Interaction effect of location × sowing time on the productivity of buckwheat.

Treatment	Parameter	Number of Seeds Per Plant		Grains of 1 Meter ² (kg)		Productivity Rate kg/Hectare	
		1st Season	2nd Season	1st Season	2nd Season	1st Season	2nd Season
Belbies City Site	Mid-Jan	29.67 b	32.78 b	0.0673 b	0.0655 b	648.76 b	627.3 b
	Mid-Nov	31.33 b	31.72 b	0.0680 ab	0.0615 b	684.46 ab	614.48 b
	Mid-Mar	37.72 a	37.83 a	0.0723 a	0.073 a	702.88 a	708.21 a
Sadat City Site	Mid-Jan	17.72 c	19.61 cd	0.0495 c	0.0472 c	481.99 c	448.23 c
	Mid-Nov	18.61 c	19.22 d	0.0482 c	0.0457 c	493.96 c	477.56 c
	Mid-Mar	21.72 c	23.22 c	0.0490 c	0.0445 c	490.67 c	444.55 c
LSD at 0.05		4.1727	3.8045	4.71×10^{-3}	7.13×10^{-3}	48.639	73.662

Different letters within the same columns indicate statistically significant differences ($p \leq 0.05$).

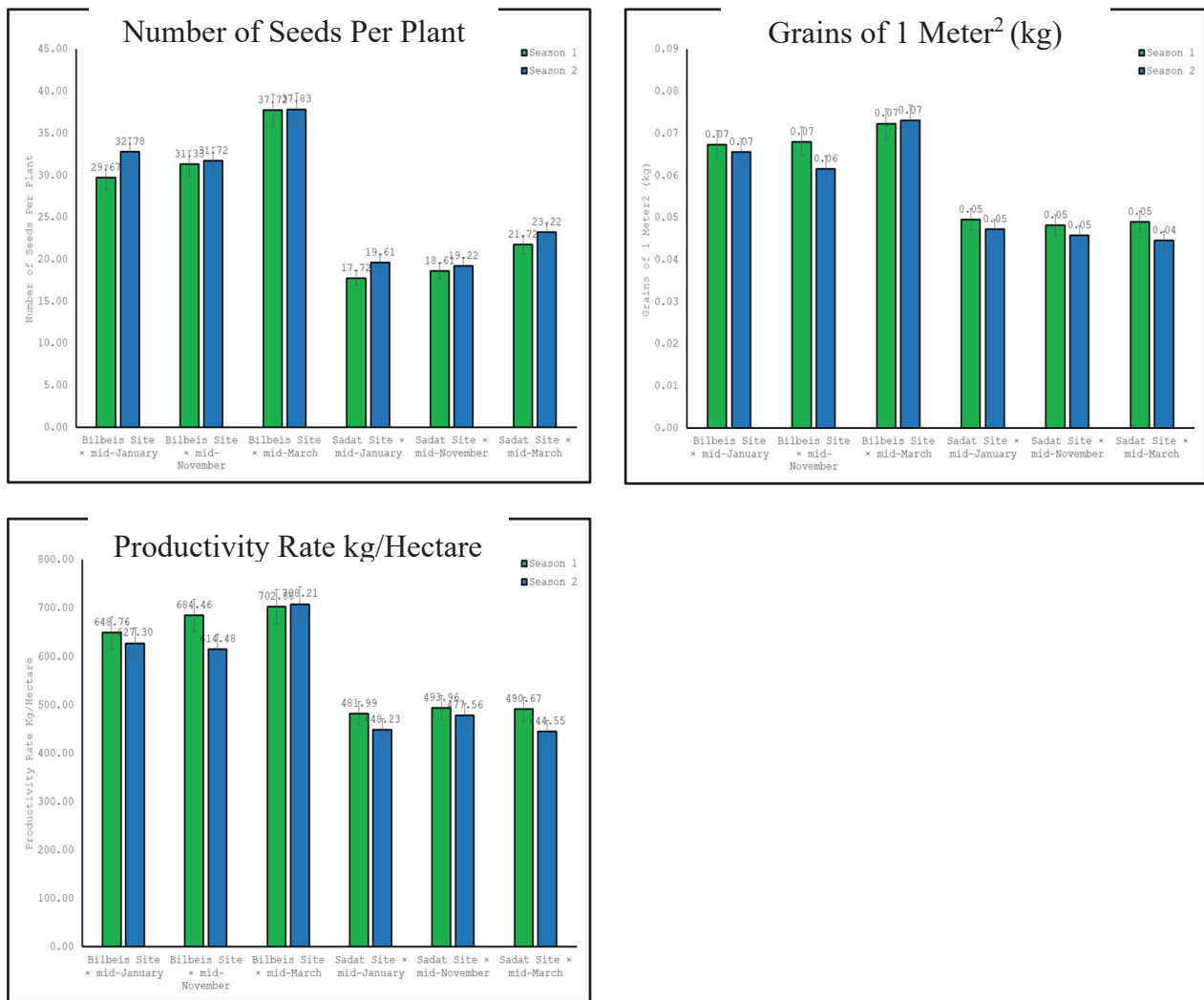


Figure 5. Evaluation of location × sowing date on the productivity of buckwheat.

These results demonstrated the significant effects of sowing time and location on the productivity of buckwheat, with mid-March sowing occurring at the Belbies City site consistently yielding the highest number of seeds, highest grain weight per square meter, and greatest productivity per hectare. Conversely, the Sadat City site had the lowest productivity across all sowing times, and the statistical analysis confirmed the robustness of these findings.

3.2.3. Interaction of Species × Sowing Date

Our obtained results in Table 9 and Figure 6 present the interaction effects of species (*Fagopyrum tataricum* vs. *Fagopyrum esculentum*) and sowing time (mid-March, mid-January, mid-November) on the productivity of buckwheat, with a focus on the number of seeds per plant, grains per square meter, and the productivity rate per hectare across two growing seasons. This analysis is structured to highlight the significant differences and trends in buckwheat productivity, providing insights into how these factors influence buckwheat productivity. For the number of seeds per plant, *Fagopyrum tataricum* produced significantly greater numbers of seeds per plant across all sowing times than did *Fagopyrum esculentum*, with the highest counts observed for mid-March sowing in both seasons (45.17 and 45.72, respectively). This indicates a clear advantage of *Fagopyrum tataricum* in terms of seed production, especially when sown in mid-March. A least significant difference (LSD) of 0.05 underscores the statistical significance of these differences. However, for grains per

square meter, *Fagopyrum tataricum* sown in mid-March also yielded the highest grain weight per square meter (0.0858 kg and 0.084 kg in the first and second seasons, respectively), significantly outperforming *Fagopyrum esculentum* sown during the same period. This finding suggested that not only the choice of species but also the timing of sowing significantly affect grain production. The LSD test results confirmed the statistical significance of these observations. However, according to the results in seeds per plant and grains per square meter, *Fagopyrum tataricum* sown in mid-March achieved the highest productivity rates per hectare (837.55 kg and 818.36 kg in the first and second seasons, respectively). In contrast, *Fagopyrum esculentum*, especially when sown in mid-March, had the lowest productivity, highlighting the substantial impact of both species and sowing time on overall productivity. The differences across these groups were statistically significant, as indicated by the LSD at 0.05. These results demonstrate the superior performance of *Fagopyrum tataricum* over *Fagopyrum esculentum* in terms of productivity, as sowing in mid-March further enhanced its productivity.

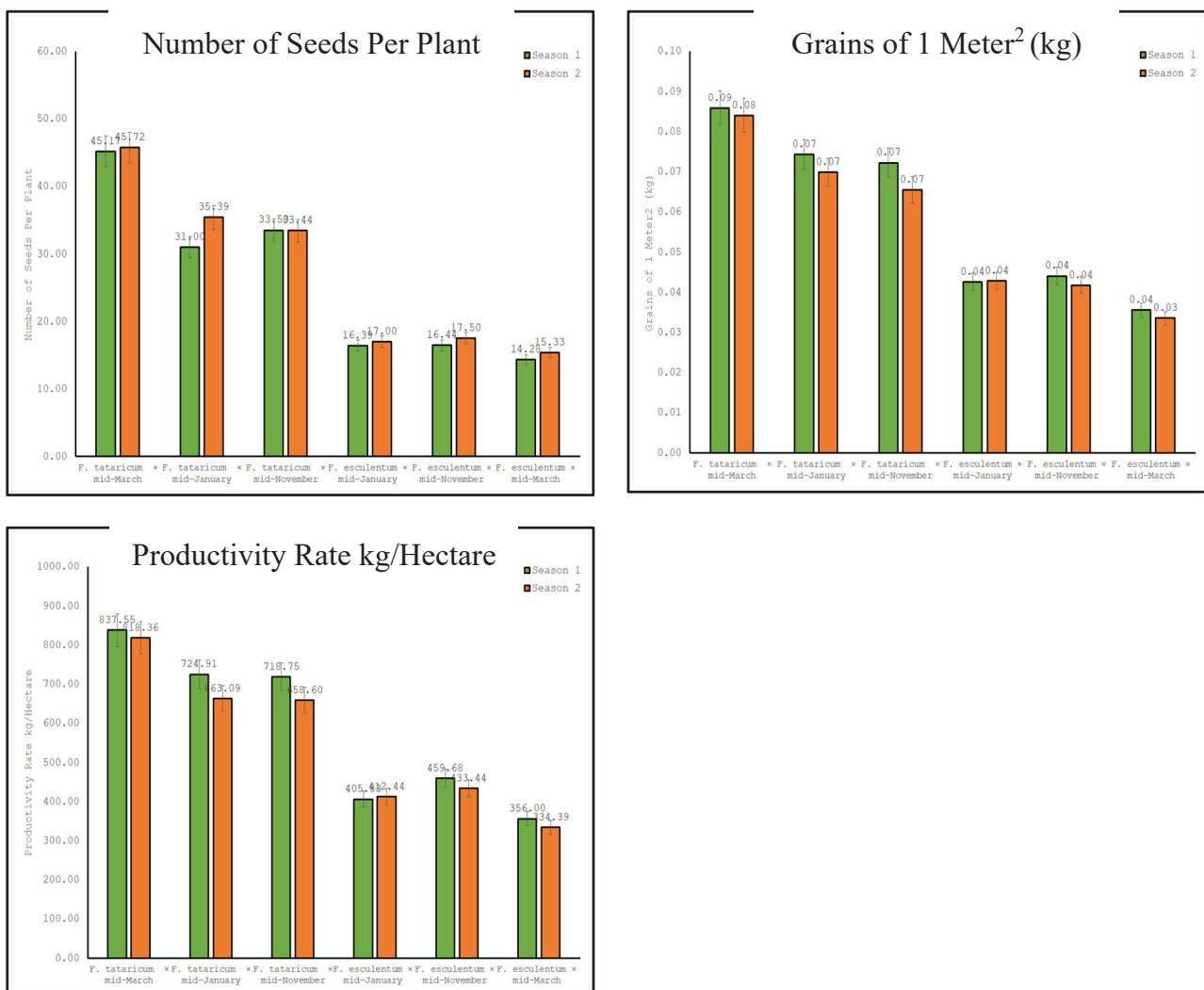


Figure 6. Evaluation of species × sowing date on the productivity of buckwheat.

Table 9. Interaction effect of species × sowing time on the productivity of buckwheat.

Treatment	Parameter	Number of Seeds Per Plant		Grains of 1 Meter ² (kg)		Productivity Rate kg/Hectare	
		1st Season	2nd Season	1st Season	2nd Season	1st Season	2nd Season
<i>Fagopyrum tataricum</i>	Mid-Mar	45.17 a	45.72 a	0.0858 a	0.084 a	837.55 a	818.36 a
	Mid-Jan	31.00 b	35.39 b	0.0743 b	0.0698 b	724.91 b	663.09 b
	Mid-Nov	33.50 b	33.44 b	0.0722 b	0.0655 b	718.75 b	658.60 b
<i>Fagopyrum esculentum</i>	Mid-Jan	16.39 c	17.00 c	0.0425 c	0.0428 c	405.83 d	412.44 c
	Mid-Nov	16.44 c	17.50 c	0.0440 c	0.0417 c	459.68 c	433.44 c
	Mid-Mar	14.28 c	15.33 c	0.0355 d	0.0335 d	356.00 e	334.39 d
LSD at 0.05		4.1727	3.8045	4.71×10^{-3}	7.13×10^{-3}	48.639	73.662

where L.S.D. = least significant difference. Different letters within the same row indicate significant differences ($p \leq 0.05$).

4. Discussion

4.1. Evaluation of the 2-Way Interaction Effect of Biotic and Abiotic Factors on the Growth of Buckwheat

The observed variations in buckwheat growth and productivity across different locations, species, and sowing dates, as indicated in the experimental outcomes, can be attributed to complex interactions among genetic, environmental, and management factors [1,6,23]. The superior vegetative growth characteristics exhibited by *Fagopyrum tataricum* (FT) in Belbies (BCS) compared to those of the Sadat City site (SCS) and *Fagopyrum esculentum* (FE) underscore the influence of both location and species on buckwheat growth. This advantage is likely due to the genetic predisposition of FT to adapt better to the environmental conditions present in Belbies, such as soil type, pH, and microclimate, which may be more conducive to its growth than the conditions in Sadat city. Furthermore, the soil and water analyses (Tables 1 and 2) provide insights into these environmental conditions, revealing differences in pH, electrical conductivity (EC), and nutrient availability between the two sites, which could significantly affect plant growth [15]. The variations in temperature, precipitation, and relative humidity (Table 3) between the locations across the seasons also influenced these outcomes. Moreover, the impact of sowing time on growth parameters highlights the significance of temporal factors on agricultural outcomes, as demonstrated by the optimal performance of Belbies plants sown in mid-March, which could be attributed to more favorable weather conditions during critical growth stages. These findings are supported by weather data, indicating that seasonal transitions and specific climatic conditions during the growth period significantly influence vegetative development [17,24]. Additionally, the interaction effect between species and sowing time, particularly the superior performance of FT over FE across all sowing times, can be explained by the inherent genetic differences between the two species. The better adaptation of FT to water stress and its drought tolerance [10] may have contributed to its greater resilience and superior growth under varying environmental conditions. The differences in genome size between these species [21,22] may also play a role in their adaptability, with FT possibly possessing genetic traits that confer an advantage under the specific conditions experienced during the experimental periods. The methodologies employed in this study, including detailed site preparation, soil and water analysis, and careful selection of sowing times and species, provided a robust framework for evaluating the interactions between genetic, environmental, and management factors in buckwheat cultivation. The use of organic manure, phosphorus fertilizer, and split applications of nitrogen fertilizer reflects contemporary agricultural practices aimed at enhancing crop productivity while considering sustainability [8,15]. The differential performance of buckwheat species across locations and sowing times is a clear indication of the complexity of genotype–environment interactions. The adaptability of *Fagopyrum tataricum* (FT) to conditions at the Belbies City site, compared to that of *Fagopyrum esculentum* (FE), whose disparity in performance at the Sadat City site mirrors previous findings [6,27], highlighted species-specific responses

to environmental conditions. This suggests that species selection cannot be universal but rather should be tailored to the specific environmental conditions of the cultivation site, taking into account the local soil, water, and climatic characteristics. The disparities in soil pH, EC, and nutrient availability between the two sites likely played a significant role in influencing plant growth and development. For instance, the relatively higher pH and EC values at the Sadat City site might have imposed additional stress on FE, which, coupled with its inherent drought avoidance characteristics, could explain its underperformance compared to that of FT, which exhibits traits of drought tolerance. These findings align with the physiological and genetic makeup of buckwheat species, which significantly influences their response to abiotic stresses [10]. The experimental design, which meticulously accounted for the variations in planting times and employed rigorous soil preparation and fertilization regimes, underscores the importance of management practices in optimizing crop yields. The application of organic manure, phosphorus, and nitrogen fertilizers, as described, likely enhanced the soil's fertility, thereby supporting the vegetative and reproductive growth of buckwheat. However, the critical role of soil health and management practices in sustainable crop production has been noted [8,15]. Furthermore, weather data analysis, which offers insights into temperature, precipitation, and relative humidity variations, provides a contextual background for interpreting the experimental results. The optimal growth and productivity observed for buckwheat sown in mid-March at the Belbies site could be attributed to the alignment of critical growth stages with favorable weather conditions, highlighting the temporal dimension of environmental suitability for crop cultivation. In summary, the findings of this study contribute to a deeper understanding of how genetic, environmental, and management factors converge to influence crop growth and productivity.

4.2. Evaluation of the 2-Way Interaction between Biotic and Abiotic Factors on the Productivity of Buckwheat

The differences in buckwheat productivity observed across various experimental conditions, including location, species selection, and sowing time, can be attributed primarily to the complex interplay of genetic, environmental, and agronomic factors. However, the significant impact of the location $\times$ species interaction particularly underscores the importance of selecting the appropriate species for specific environmental conditions, as *Fagopyrum tataricum* exhibited greater productivity in Belbies than in Sadat due to its genetic adaptability to local environmental and soil conditions [5]. Moreover, its inherent genetic traits, such as increased tolerance to local abiotic stresses indicated by soil pH, EC, and soil texture characteristics, further contributed to its superior performance in Belbies. Conversely, *Fagopyrum esculentum* showed reduced productivity across both locations, indicating its possibly lesser adaptability to the given environmental conditions or its specific genetic makeup, rendering it less efficient under the tested conditions [6,27]. Furthermore, the interaction between location and sowing date highlights the critical role of sowing timing in optimizing buckwheat productivity, with mid-March sowing in Belbies achieving the highest productivity due to the optimal alignment of the sowing date with favorable environmental conditions, such as temperature and humidity, conducive to buckwheat growth. This is supported by weather data analysis, where temperature and precipitation patterns likely favored the mid-March sowing period, resulting in enhanced growth and productivity [12,20]. Additionally, the interaction between species and sowing date provides insights into how genetic factors combined with agronomic practices influence crop outcomes, with *Fagopyrum tataricum* exhibiting superior performance after mid-March sowing, emphasizing its adaptability to the climatic conditions that prevailed during this period. This adaptability, possibly due to its genetic traits favouring growth under specific environmental conditions experienced in the spring season in Egypt, further accentuates the need for species-specific management practices to optimize productivity. The observed variances in productivity related to sowing time and species selection underscore the complex genotype-by-environment interactions that influence crop performance [8]. Moreover,

comprehensive soil and water analyses were conducted at the beginning of each experimental season, and the physical and chemical properties of the soil and irrigation water quality were recorded; these analyses directly impacted plant growth, development, and ultimately productivity. The suitability of the soil and water properties in Belbies for buckwheat cultivation, especially for *F. tataricum*, may have contributed to the higher productivity observed at this location. Additionally, the methodological approach, including the use of organic manure, phosphorus fertilizer, and nitrogen fertilizer, likely provided an optimal nutrient regime that supported the growth and productivity of buckwheat plants under the tested conditions. The strategic application of fertilizers, based on the specific growth stages of buckwheat, enhances nutrient availability during critical periods of growth and development, contributing to the observed differences in productivity across different sowing times and species. In conclusion, the results of this study elucidate the intricate relationships among genetic, environmental, and management factors in determining crop productivity. The adaptability of *Fagopyrum tataricum* to the specific conditions in Belbies, especially when sown in mid-March, highlights the potential for optimizing buckwheat cultivation through careful selection of species and sowing times that align with local environmental conditions. These findings contribute to the broader understanding of sustainable agricultural practices that can enhance crop resilience and productivity in the face of changing climatic conditions, providing valuable insights into the development of sustainable agricultural practices that leverage genetic diversity and environmental management to optimize crop productivity [22,25,28].

5. Conclusions

This research highlights critical findings on buckwheat cultivation in Egypt from three interactions: location $\times$ species, location $\times$ sowing date, and species $\times$ sowing date. The study demonstrated that *Fagopyrum tataricum* significantly outperformed *Fagopyrum esculentum*, particularly when sown in mid-March. It also showed a significant increase by planting it in the Belbies City site, with productivity reaching up to 859 kg/ha. This underscores the adaptability of this species and the importance of choosing the right sowing dates. Additionally, the interaction between location and sowing date revealed that mid-March sowing in Belbies yielded the highest productivity, emphasizing the impact of environmental conditions on crop growth. The study's insights into these interactions offer valuable guidance for optimizing buckwheat cultivation in Egypt, contributing to the development of sustainable agricultural practices in arid and semiarid regions.

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Article

Factors Influencing the Accumulation of Free Asparagine in Wheat Grain and the Acrylamide Formation in Bread

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Abstract: Asparagine is one of the precursors of acrylamide that can form during bread production. The aim of this work was to determine the effect of genotype, environment, sulfur fertilization, and the interaction of those factors on the asparagine content, technological value of wheat, and acrylamide level in bread. The research material consisted of five wheat cultivars grown in two locations in Poland with nitrogen fertilization of 110 kg ha^{−1} and sulfur fertilization of 30 kg ha^{−1}. The standard ISO method for analyzing the milling and baking properties of wheat was used. The UHPLC-MS/MS method for analyzing the amino acids and the GC/MS method for acrylamide in bread were implemented. The analysis of variance results indicated that the location influenced the total variance in the measured asparagine content and quality of wheat the most, followed by the cultivar and then by the interaction between the environment and cultivar. Sulfur fertilization had no significant effect on the asparagine content, but slightly lowered the gluten quality and loaf volume of bread. However, sulfur fertilization in connection with the cultivar characterized by low starch damage had a positive effect on lowering the acrylamide in bread. Asparagine content in wheat and acrylamide in bread varies mostly depending on cultivar and environment.

Keywords: asparagine; acrylamide; baking value; starch damage; sulfur fertilization; wheat cultivars

1. Introduction

Wheat is known for being one of the most important crops grown in the world. In 2021, the total global production equaled 770 million tons, with an average yield of 3.6 t ha^{−1} and a harvested area of about 220 million ha [1]. Bread is a staple food for a large proportion of the world's population. Wheat bread, without a doubt, is an integral part of the daily diet and delivers not only carbohydrates and protein but also crucial health-promoting ingredients, especially when consumed as whole grain [2]. However, in the process of baking bread, acrylamide is formed, which may be a health risk, because acrylamide has been classified as being probably cancerogenic to humans (Group 2A). It has also been shown to have neurological and reproductive effects at high doses in rodent toxicology studies. Acrylamide forms mainly from free asparagine and carbonyl sources as part of the Maillard reaction [3]. Numerous food processing parameters, including temperature, formulation, and cooking time, affect acrylamide formation from asparagine. Acrylamide can be found in most cereal products, including bread and breakfast cereals, however, in particularly high concentrations in crispbread, cookies, and gingerbread [4].

A number of investigations focused on strategies to minimize the levels and the formation of acrylamide in wheat products. Based on the results, the reduction in heat and the addition of specific ingredients—for example, cysteine or asparaginase—alongside

prolonged fermentation time is proposed [5]. However, all technological efforts to reduce acrylamide might be in vain if unsuitable raw material is used. Amrein et al. [6] proved that in French fries and potato chips, acrylamide content is dependent on the potato cultivar. Additionally, several studies showed that cultivars play a very important role in acrylamide content in baking products. Claus et al. [7] described that acrylamide content in wheat cultivars varied in the range of 8.7 to 24.9 mg/100 g. Also, Taeymans et al. [8] reported a range of 7.4 to 66 mg/100 g. Claus et al. [7] stated that, generally, wheat varieties belonging to the good quality category of wheat—E and A—show higher acrylamide levels than cultivars belonging to the low category—B and Ck. However, these authors also note that, among high-quality category A cultivars, it is possible to find cultivars with a high crude protein content and relatively low acrylamide concentration. The differences result from different amounts of asparagine in the grain of wheat [7,9]. Rapp et al. [10] showed that the mean asparagine content ranged from 143.25 to 392.75 mg/kg for the different wheat cultivars. Malunga et al. [11] found that the asparagine content of Canadian wheat varied from 302 to 965 and 116 to 336 mg kg⁻¹ for whole meal and white flour, respectively. Genotype was a factor that had a significant effect on the asparagine content, suggesting that breeding strategies are an interesting avenue to pursue in order to produce cultivars with lower levels of this amino acid.

For this reason, focusing studies on the reduction of the precursors of acrylamide in wheat grains by lowering the asparagine content seems like the best way forward. Asparagine accumulates under stress conditions as a biological response to the restriction of protein synthesis. Stressful conditions can be caused by drought, salt in the soil, pathogen attack, toxicity, solar radiation, or nutrient deficiencies [9,12,13]. Asparagine often plays a pivotal role in the active site of enzymes [14,15]. Interestingly, the biosynthesis of all essential amino acids can be achieved from asparagine as a donor of fixed nitrogen. High free asparagine concentrations in grain indicate a poor efficiency of nitrogen utilization, defined as the conversion of assimilated nitrogen to grain protein, resulting in lower-quality grain. Asparagine content in flour and dough significantly affected acrylamide content [7]. Furthermore, it was found that asparagine concentration in grain was affected by both genotype and production environment [7,9,11,13]. In the Claus et al. [7] study, both asparagine and acrylamide content were shown to be dependent on weather conditions during the growing season, especially temperature.

Nitrogen is a vital component of amino acids; therefore, nitrogen fertilization has a strong impact on crude protein and free asparagine contents in flour [16,17]. Higher doses of nitrogen influenced the increase of asparagine in wheat grains [7,18]. In addition, sulfur was shown to have an important role in acrylamide formation and its precursors [16,17]. Curties and Halford [16] demonstrated a significant effect of sulfur fertilization on asparagine content in wheat grain. In conditions of sulfur deficiency in the soil, the amount of asparagine in grain was 26 mmol/kg (3435 mg/kg), while under sufficient sulfur conditions, the amount was 1.6 mmol/kg (211 mg/kg). Halford et al. [17] showed that even flours from wheat grown in the field with an intermediate level of sulfur application (10 kg sulfur per hectare) contained more acrylamide after processing than flours from wheat grown with the application of 40 kg sulfur per hectare.

The use of wheat for consumption requires appropriate quality characteristics that are important from a bakery's point of view. Wheat baking quality parameters depend on cultivars, weather conditions, and fertility. Sulfur and nitrogen fertilizer application on winter wheat can have many benefits. Nitrogen is an essential nutrient important for wheat growth and protein biosynthesis. Low nitrogen availability can limit crop growth, yield, and quality [19]. Adequate sulfur is also required for the proper growth and development of wheat, sulfur being essential for the biosynthesis of amino acids, proteins, and chlorophyll, in particular [20]. Sulfur is a key element of enzymes involved in nitrogen metabolism [21–23]. Moreover, sulfur deficiency can decrease nitrogen assimilation [24]. Without an adequate supply of sulfur, wheat is unable to reach its full yield potential and cannot efficiently utilize nitrogen for protein biosynthesis [25]. Therefore, nitrogen use efficiency and crop

yields are increased when nitrogen and sulfur are applied together [24,26,27]. Two critically important amino acids, methionine and cysteine, contain sulfur. Methionine is the initiating amino acid in the synthesis of all proteins, and cysteine plays a pivotal function in protein folding [28]. Nitrogen and sulfur deficiency are detrimental to baking quality due to their influence on the formation of disulfide bonds formed from the sulfhydryl groups of cysteine. Such bonds are important because they influence the viscoelasticity of dough [29].

Sulfur can be deposited in the soil in a variety of ways. According to Ref. [30], the US Federal Clean Air Act revisions introduced in 1990 have strongly improved air quality by regulating power plant air emissions. As a result, sulfur emissions have decreased by 80%, resulting in the reduction of sulfur deposition along with lower sulfur deposition. Lower deposition combined with a lack of sulfur fertilization results in soil sulfur deficiency throughout the world [31]. Although the topic of asparagine and acrylamide has been discussed for several years, the number of publications on the interactions between genotype, growing location, and sulfur, as well as their impact on the asparagine content and baking quality of wheat, is still limited and requires supplementation. We hypothesize that the use of sulfur will reduce the amount of free asparagine and affect the baking quality of winter wheat, but the effect of sulfur on reducing asparagine will depend on the cultivar.

Another factor that we chose to consider in this research is the influence of starch damage in wheat flour on the formation of acrylamide in bread. Damaged starch formation occurs during the milling of grain due to the mechanical shear between the mill rolls [32]. Among the factors affecting the amount of damaged starch in the flour are wheat grain hardness, wheat tempering conditions, and the setting of the mill (i.e., roll diameter and speed ratio, pressure, and gap size between the reduction rolls, milling time, and roll temperature) [32,33]. Starch damage strongly affects the behavior of dough during processing (e.g., water absorption, gelatinization, and rheological properties), as well as the quality of the finished product (e.g., color, shelf life) [32]. However, levels of damaged starch in the work of Wang et al. [34] and Mulla et al. [35] for wheat flours showed a strong positive correlation with acrylamide formation in bread. An increase in starch damage was also correlated with reduced sugar content in flour [34,35]. Another hypothesis of our work was to prove that starch damage might influence the acrylamide content in bread.

Therefore, the goal of this study was to evaluate the influence of wheat cultivars, sulfur fertilization, and growing location impact on the asparagine and acrylamide content and baking quality of winter wheat.

2. Materials and Methods

Plant material. This research work concerned five winter wheat cultivars representing two technological groups approved by the Polish National List of Agricultural Plant Varieties issued yearly by COBORU (The Research Center for Variety Testing). Group A comprises quality bread cultivars, namely ‘Hondia’ and ‘Pokusa’. Group B comprises bread cultivars ‘Belissa’, ‘Hybery F₁’, and ‘Bonanza’.

Field trials. Field trials were conducted during the 2020–2021 and 2021–2022 growing seasons at the Experimental Stations in Wielichowo (52°07′ N 16°21′ E) and Werbkowice (50°45′ N 23°45′ E), Institute of Soil Science and Plant Cultivation—State Research Institute (IUNG-PIB) Puławy, Poland. The soil in Werbkowice was typical chernozem, class I soil categorized as a very good wheat soil complex that is characteristic of the region. In Wielichowo, the soil was pseudopodsolic, class Iva, categorized as a very good rye complex. Winter rapeseed was the fore crop. The experiment was established using the long strip method. The first factor was the cultivar (‘Hondia’, ‘Pokusa’, ‘Belissa’, ‘Hybery F₁’, and ‘Bonanza’), and the second factor was sulfur fertilization. Plots with no sulfur fertilization were used as control objects. Before the start of the trial, the soil was analyzed down to a depth of 30 cm. In Werbkowice, the soil was characterized by 7.9 pH_{KCl}, and the soil fertility indicators were 19.5 mg of K₂O, 10.2 mg of P₂O₅, and 0.16 mg of S per 100 g of soil, and 12.5 mg of Mg per 100 g of soil. In Wielichowo, the soil was characterized by 6.2 pH_{KCl}, and the soil fertility indicators were 19.2 mg of K₂O, 25.3 mg of P₂O₅, and 0.78 mg of S per

100 g of soil, and 6.4 mg of Mg per 100 g of soil. In both experimental stations, the nitrogen in the planned total dose of 110 kg ha⁻¹ was applied in divided doses. The first dose of 60 kg ha⁻¹ was applied after resumption of spring vegetation and the second dose of 50 kg ha⁻¹ was applied at the stem elongation stage. One dose of sulfur was 30 kg ha⁻¹ and was applied after the resumption of spring vegetation together with nitrogen fertilization. Winter wheat was sown on 5 October (Werbkowice) and 28 September (Wielichowo) in an optimal sowing term at a seed rate of 450 germinable kernels per m². The area of the harvested plot was 0.7 ha. Pre-sowing practices, pre-sowing plowing, and mineral fertilization (NPK) were accomplished before sowing. A 350 kg ha⁻¹ dose of Polifoska 6-NPK (6 20 30) fertilizer was applied, which was at a concentration equivalent to 21 kg of N, 70 kg of P₂O₅, and 105 kg of K₂O per ha. All winter wheat cultivars were chemically protected against pests. Weed control consisted of 1.0 L ha⁻¹ of Kantor Forte 195SE and 0.5 L ha⁻¹ Puma Universal 0.69 EW. Disease control consisted of 1.0 L ha⁻¹ of Tilt Turbo at the flag leaf stage and 2.0 L ha⁻¹ of Adaxar Plus after blossoming. Pest control consisted of 0.3 L ha⁻¹ of Fury 100 EW at the flag leaf stage. The crop was harvested at full maturity on 2 August 2021 and on 28 July 2022.

Weather conditions during the growing season of winter wheat were assessed on the basis of the Hydrothermal Coefficient of Selyaninov (HTC). During the growing seasons from February to August, the HTC was 1.04 (2021) and 1.14 (2022) in Werbkowice and 1.32 (2021) and 1.28 (2022) in Wielichowo. This indicates that in Werbkowice, the year was dry, while in Wielichowo, it was optimal. Particularly large differences occurred in July during the grain ripening period. In Werbkowice, the HTC was 0.54 (2021) and 0.62 (2022), while the HTC in Wielichowo was 1.43 (2021) and 1.32 (2022). Therefore, during the grain ripening period, there was a drought in Werbkowice, while in Wielichowo, there were optimal weather conditions.

Because of a comparable impact of factors (cultivar and sulfur fertilization) in both years and taking into account the large amount of work needed to determine milling tests and the assessment of rheological characteristics of dough and baking tests, the cultivar samples from both years were mixed appropriately for further research.

Technological quality analysis. Test weight (ISO 7971-3:2019) [36], protein content (ISO 20483:2013) [37], gluten quantity (ISO 21415-2:2015) [38], Zeleny sedimentation index (ISO 5529:2007) [39], and ash content (ISO 2171:2007) [40] were evaluated to determine the baking value of five wheat cultivars. Grain samples were ground to whole meal flours using hammer mill FN 3100 (Pertin Instruments AB, Hägersten, Sweden) for protein and gluten content analyses. The Sedimat laboratory mill (Brabender GmbH & Co. KG, Duisburg, Germany) was used to prepare samples for the Zeleny sedimentation test.

Amino acid analysis. Sample preparation. Free amino acid content was analyzed based on the method described by Świder et al. [41] with slight modifications. In brief, 2 g of a sample was placed in a centrifuge tube and spiked with internal standard (1.7—diaminoheptane solution, Merck, Darmstadt, Germany). In order to perform extraction, 40 mL of trichloroacetic acid (5% solution, Avantor Performance Materials, Gliwice, Poland) was added and the sample was shaken thoroughly. After centrifugation (10,000 × g for 10 min.), 100 µL of the supernatant was mixed in a 15 mL volume centrifuge tube with 2.5 mL of di-sodium tetraborate (3% water solution, J.T. Baker, Pol-Aura, Gliwice, Poland) and 2.5 mL of dansyl-chloride (20 mMol in acetonitrile, abcr GmbH, Karlsruhe, Germany) to derivatize the analyzed compounds. Derivatization was performed by shaking in a water bath at 40 °C for 1 h. Then, 10 µL of formic acid (98–100% purity, Avantor Performance Materials, Gliwice, Poland) was added to stop derivatization. The sample was filtered into a chromatographic vial using a syringe filter (pore size: 0.22 µm, Captiva Econofilters, Agilent, Santa Clara, CA, USA). Amino acid standards were supplied by Merck (Darmstadt, Germany) and their assays were as follows: asparagine, arginine, aspartic acid, threonine, valine, methionine, phenylalanine, leucine, isoleucine, lysine, tyrosine, histidine ≥98%, glutamine, proline, ornithine, glutamic acid, and serine ≥99%.

Chromatographic analysis. UHPLC-MS/MS (ultra-high performance liquid chromatography coupled with a high-resolution mass spectrometer) (Q Exactive Orbitrap Focus MS Thermo Fisher Scientific, Waltham, MA, USA) analysis was performed to determine free amino acid concentration. The compounds were separated on the Cortecs UPLC C18 2.1 × 100 mm, 1.6 µm column (Waters, Milford, MA, USA). Phase A consisted of water:ACN (90:10)/0.1% FA/5 mM ammonium formate, and phase B contained can:water (90:10)/0.1% FA/5 mM ammonium formate. LC-MS-grade water and LC-MS-grade acetonitrile were supplied by Witko (Łódź, Poland), and formic acid 98–100% and ammonium formate ≥97% for LC-MS were supplied by Chem-Lab (Zedelgem, Belgium). The following liquid phase gradient at the flow rate 0.3 mL/min was applied: A:B (%) 0–2 min.—90:10—waste, 2–22 min.—0:100, 22–25 min.—0:100, 25–26 min.—90:10, 26–28 min.—90:10. MS was equipped with heated electrospray ionization (HESI) source. Analysis was performed in positive polarization mode with an injection volume of 2.5 µL. The detailed MS parameters were set as follows: spray voltage: 3 kV, capillary temperature: 256 °C, sheath gas flow rate: 48, auxiliary gas flow rate: 11, sweep gas flow rate: 2, probe heater temperature: 413 °C, S-lens RF level: 50, resolution: 70,000 in simultaneous scan and 35,000 in all ion fragmentation. Acquisition and analysis of the data were performed using Xcalibur 4.2.47 software.

Milling and baking quality analysis. Wheat grain samples weighing approx. 2.5 kg each were moistened to 16% with water. After resting for 16 ± 1 h, the wheat samples were milled using an MLU-202 laboratory flour mill (Bühler AG, Uzwil, Switzerland) to obtain flour for the measurement of the rheological characteristics of dough by mixolab acc. to ISO 17718:2013 [42] (KPM Analytics, Chopin Technologies, Villeneuve-la-Garenne, France) and to perform laboratory baking test. Flour yield was in the range of 68.1 to 75.4%. Damage starch of flour was analyzed according to ISO 17715:2013 [43] by Chopin SDmatic (KPM Analytics, Chopin Technologies, Villeneuve-la-Garenne, France).

Baking test. The wheat flour samples were evaluated by a standard baking test for pan bread [44]. The dough was prepared by one-stage method at 28–30 °C by mixing flour (100%), water (acc. To water absorption specified by Mixolab + 3%), yeast (3%), and salt (1%) using a laboratory mixer (KitchenAid, Benton Harbor, MI, USA). The dough in bulk was fermented for 60 min in a proofing chamber at 30 °C/75% RH. Then, after 30 min, kneading by hand was performed. The dough was next divided into three dough pieces with a mass of 250 g each that were molded round and placed in baking pans in a proofing chamber for 38–47 min (the time needed for optimal dough development). The loaves were baked in an oven (230 °C, 30 min) (Piccolo Wachtel Winkler, Wachtel ABT GmbH, Pulsnitz, Germany). The bread samples were assessed after 20 ± 1 h of cooling for loaf volume, bread crumb hardness (Instron 1140, Instron, Norwood, MA, USA), and acrylamide content using GC/MS after bromination [45]. Anion exchange solid phase extraction combined with octadecyl silica was used to perform sample cleanup. Based on validation experiment results, it was confirmed that the method performs well at low acrylamide concentration levels with LOQ values at 10 µg kg⁻¹, and recovery relative standard deviation below 6%. The validation process was performed according to ISO/IEC 17025:2017 [46] requirements. Detailed statistical parameters of the described method have been reported by Roszko et al. [45].

Statistical analysis. The results were statistically analyzed using the three-way analysis of variance (ANOVA) with subsequent Tukey's HSD test with the significance level of $p < 0.05$. The three main factors were localization, sulfur fertilization applied, and the wheat cultivar used. A principal component analysis (PCA) was carried out on the average results of each cultivar. Data were analyzed using Statistica v. 13 software (Tibco Inc. Palo Alto, CA, USA).

3. Results and Discussion

3.1. Technological Value of Wheat Samples

The tested wheat samples were varied in terms of basic quality characteristics used in assessing suitability for processing grain for consumption purposes. The analysis of

variance results (Table 1 and Table S1) indicated that the localization had the biggest influence on the total variance in the measured technological parameters of wheat grain, followed by cultivar, and then by the interaction between the environment and cultivar.

Table 1. F values calculated in the three-way ANOVA made for the qualitative parameters of grain of the examined wheat samples.

Parameter	Sources of Variation						
	Localization (A)	S Fertilization (B)	Cultivar (C)	A × B	A × C	B × C	A × B × C
Test weight	205.30 **	0.22	28.85 **	2.33	12.03 **	0.85	2.82
Protein content	2695.88 **	1.02	78.34 **	2.61	162.79**	3.09 *	6.92 **
Gluten content	2160.08 **	12.51 **	92.71 **	2.52	84.61 **	0.60	1.56
Zeleny index	84.70 **	0.42	9.97 **	0.58	14.52 **	0.91	0.74
Ash content	15.80 **	0.00	1.54	0.10	1.71	0.20	0.42
Asparagine	3418.61 **	1.50	43.33 **	27.35 **	17.04 **	2.82	4.88 **

Significant at: * $p < 0.05$, ** $p < 0.01$.

Wheat samples grown on location in Wielichowo were characterized by the highest average value of the test weight (74.0 kg hl^{-1}), which is an important predictor of the flour extraction rate for wheat (Table 2). However, these samples showed lower protein and gluten content (13.8% and 27.8%, respectively), with lower gluten quality (Zeleny index) and ash content than wheat grown in the Werbkowice location. Wheat grain samples from the Werbkowice location were also characterized by the highest value of asparagine content (525.1 mg kg^{-1}). Field location significantly influences the protein content and asparagine content. The analysis of variance results (Table 1) indicated that localization predominated (91.1%) the total variance in the measured free asparagine concentration in tested whole meal samples. The genotype, as well as the interaction localization and cultivar, contributed only 4.6 and 1.8%, respectively. For the wheat cultivars tested in this study, fertilization had the least impact on free asparagine content, contributing only 0.4% to the variance in free asparagine concentration. Similar to the result of Xie et al. [47], the interaction between genotype and fertilization and the interaction between localization and fertilization contributed less than 2% to the total variance.

The protein content of grain in the Werbkowice location was, on average, 2.5 p.% (percent points) higher than in Wielichowo. Additionally, we found interaction cultivar × localization in protein content (Table 1). In Wielichowo, sulfur fertilization did not cause significant changes in protein content, while in Werbkowice, sulfur fertilization caused an increase in protein content in the ‘Hybery F₁’ and ‘Bonanza’ cultivars (Table S2). For the cv. ‘Pokusa’ grown in Werbkowice, sulfur fertilization resulted in a reduction of the asparagine content in the grain. However, in Wielichowo, sulfur caused a decrease in the asparagine content in the seeds of the ‘Belissa’ and ‘Bonanza’ cultivars. Previous research also investigated environmental factors and agronomic differences that significantly influenced the free asparagine content in wheat grain [48]. They attempted to find answers to a question of how different environmental conditions and certain agronomic practices impact asparagine content in grain. Research from Malunga et al. [49] also showed that the asparagine concentration was significantly influenced by location and was responsible for 80% of the variation compared to 13% for the cultivar factor. Tafuri et al. [50] proved that several Italian cultivars seemed to be highly influenced by the environment, whereas others indicated relative stability in free asparagine content across locations and years. Xie et al. [47] stated that cultivating wheat in suitable environments, together with the selection of wheat genotypes with lower potential to form free asparagine content, is the most effective proposed action to control free asparagine content in Canadian wheat.

Table 2. Technological value of grain of wheat cultivars tested in the experiment.

Factor	Test Weight (kg hl ⁻¹)	Protein Content (N × 5.7) (%d.m.)	Gluten Content (%)	Zeleny Index (cm ³)	Ash Content (% d.m.)	Asparagine Content (mg kg ⁻¹)
Localization (A)						
Wielichowo	74.0 ^a	13.8 ^b	27.8 ^b	55 ^b	1.59 ^b	276.2 ^b
Werbkowice	71.5 ^b	16.3 ^a	34.2 ^a	63 ^a	1.68 ^a	525.1 ^a
Fertilization (B)						
N + 0S	72.7	15.0	30.7 ^b	59	1.63	392.4
N + 30S	72.8	15.1	31.2 ^a	59	1.63	408.9
Cultivar (C)						
Hondia	73.6 ^{ab}	14.6 ^d	29.9 ^d	56 ^b	1.60	378.3 ^{cd}
Pokusa	72.9 ^{bc}	15.8 ^a	33.4 ^a	63 ^a	1.65	397.1 ^c
Belissa	71.3 ^d	15.3 ^b	31.4 ^b	60 ^{ab}	1.59	426.8 ^b
Hybery F ₁	73.8 ^a	14.6 ^d	29.7 ^d	56 ^b	1.66	363.5 ^d
Bonanza	72.2 ^c	15.0 ^c	30.7 ^c	58 ^b	1.64	437.4 ^a

a, b, c, d—values marked with the same letters do not differ significantly at $p < 0.05$ according to Tukey's multiple test.

In our study, the applied form of sulfur fertilization had no significant effect on the mentioned technological grain parameters, except gluten content (Table 1). Similar to the results of Klikocka et al. [51], the application of sulfur caused a slight positive effect on the increase in gluten content. Sulfur addition had no negative effect on the grain characteristics for processing for baking purposes. In our results, the asparagine content was higher in low-sulfur soil (Werbkowice) compared with high-sulfur soils (Wielichowo). Our results are consistent with the research of Wilson et al. [52] and Muttucumaru et al. [53]. In the research of Wilson et al. [52] and Muttucumaru et al. [53], free asparagine content in grain cultivated in low-sulfur soils was strikingly high—up to 30 times more than that cultivated in soil with normal sulfur levels. Wilson et al. [52] showed that low nitrogen use efficiency genotypes produced grain with greater asparagine content than high nitrogen use efficiency genotypes under sulfur-deficient conditions, compared to other research. Despite that, sulfur application reduces asparagine content to baseline levels. According to Xie et al. [47], fertilization had the least impact on asparagine content. Sulfur fertilization strategies for reducing free asparagine concentration in wheat were not always effective. In the research conducted by Stockmann et al. [15] in non-sulfate-deficient soils, sulfur fertilization within a conventional farming system did not influence the protein content and free asparagine amount significantly. Sufficient sulfur accumulation in the grains is presumably supplied from the available sulfur in the soil during the grain-filling period [15,54]. However, different accumulation of asparagine content in different cultivars, as a result of sulfur fertilization, was observed in both locations. The asparagine content in Werbkowice was found to be lower by 16.3 mg kg⁻¹ for cv. 'Pokusa' and higher for cv. 'Hondia', 'Belissa', 'Hybery F₁', and 'Bonanza' (on average, 52 mg kg⁻¹) for grain with N + 30S treatment compared to N treatment. For the Wielichowo location, the differences were from −34 mg kg⁻¹ for cv. 'Belissa' to +9 mg kg⁻¹ for cv. 'Pokusa' (Table S2).

Tested wheat cultivars were characterized by high protein and gluten content with good potential for bread making. Among analyzed wheat cultivars, cv. 'Pokusa' was characterized by the highest value of wheat for baking purposes with the highest value of protein and gluten content and the best gluten quality determined by the Zeleny index (Table 1). The 'Hondia' and 'Hybery F₁' cultivars, despite the highest test weight, showed a slightly lower baking value.

The free asparagine content in wheat varied widely both within cropping systems and cultivars [15]. This was also confirmed in this study as the asparagine content in tested wheat samples ranged from 235.6 (cv. 'Hondia', N fertilization) to 587.9 mg kg⁻¹ (cv. 'Bonanza', N + 30S fertilization) (Table S2). Therefore, wheat grown in Poland has

similar asparagine levels to wheat cultivated in other parts of the world, i.e., in Canada where asparagine content ranges from 302.2 to 700.3 mg kg⁻¹ for whole meal flour [11], and was lower than those of whole meal obtained from hard red spring cultivated in North Dakota and Nebraska, USA (357–1037 mg kg⁻¹ [55] and 200–110 mg kg⁻¹ [13], respectively). The asparagine content of wheat grown in Europe was in a wider range (320–1560 mg kg⁻¹) [56], while that of wheat cultivated in Australia was relatively lower (137–437 mg kg⁻¹) [57]. Free asparagine content in a total of 54 bread wheat cultivars for the Italian market ranged from 0.55 to 2.84 mmol kg⁻¹ dry matter (73 to 375 mg kg⁻¹) [50]. Malunga et al. [49] suggested that breeding strategies should aim to identify cultivars that are low asparagine accumulating and are stable across different growing environments. These observations suggest that five tested wheat cultivars are not stable in diverse environments. However, since only two crop years and two growing locations were used in this research, multiple years, locations, and cultivars are required to understand the differences in polish conditions. In an ideal scenario, cultivars in which the influence of localization on the asparagine content is not significant would be found.

Asparagine content was negatively correlated with test weight ($r = -0.831$) and positively correlated with protein content ($r = 0.855$), Zeleny index ($r = 0.706$), and ash content ($r = 0.614$) (Table S3). Higher protein content may increase levels of free asparagine. Stockmann et al. [15] reported a poor relationship between protein and free asparagine for nitrogen trials, whereas the conventional sulfur trial showed a good correlation for both traits. Ohm et al. [55] found significant but low simple linear correlations between free asparagine content and test weight, as well as wheat protein content and bread volume, suggesting that high asparagine content might be associated with good bread quality.

The significant interaction between localization and fertilization was stated only for asparagine content (Table 1). The interaction between localization and cultivar had a significant impact on test weight, protein content, gluten content, Zeleny index, and asparagine, contributing 11.8, 17.5, 11.6, 27.6, and 1.8% of the total variance, respectively (Table S1). The interaction between fertilization and cultivar was found only for protein content, whereas interactions between all three tested factors—localization $\times$ fertilization $\times$ cultivar—were found only for protein content and asparagine content and contributed only 0.7 and 0.5% of the total variance, respectively.

3.2. Quality Characteristics of Wheat Flour Samples Obtained from Tested Cultivars

Flour samples from wheat cultivars obtained in laboratory milling were evaluated in terms of quality parameters describing the properties of both protein and starch complexes. Both indirect and direct methods were used for conducting laboratory baking tests.

The ANOVA results (Tables 3 and S4) indicated that both localization and cultivar, as well as the interaction between these two factors, had the greatest share in the total variance in the measured quality parameters that characterized the protein complex of flour. Localization had the greatest share in the total variance of such quality parameters as protein content (63.8% in the total variance) and protein weakening in points C2 (78.9%) and C18 (81.3%), respectively (Table S4). For the wheat samples tested in this study, the cultivar had the second strongest impact on such parameters as water absorption, development time T1, gluten index, gluten content, and stability, contributing to 58.5%, 47.0%, 43.3%, 40.6%, and 30.7% of the variance in their concentration, respectively.

Results of the quality characteristics of the protein and starch complex of flour are presented in Table 4. Flour samples obtained from grain from the Werbkowice localization were characterized by significantly higher protein and gluten content than samples obtained from the Wielichowo location. Even though the quality of gluten measured by the gluten index was slightly lower, it still met the requirements for good baking value of flour for bread production [58]. Protein quantity and quality influenced the water absorption of flour and the technological properties of dough measured by mixolab. Flours from grain samples from the Werbkowice location, which were also characterized by higher ash content, showed higher water absorption values (on average 57.3%) compared to

Wielichowo location (54.4%). However, the lowest quality of gluten samples from the Werbkowice location caused significantly lower values of dough stability and torque in points C2 and C₁₈.

Table 3. F values calculated in the three-way ANOVA made for the qualitative parameters of the flour obtained from the grain of the examined wheat samples.

Parameter	Sources of Variation					
	Localization (A)	S Fertilization (B)	Cultivar (C)	A × B	A × C	B × C
Protein content	530.45 **	1.25	23.05 **	1.25	50.07 **	0.62
Gluten content	93.06 **	0.45	34.22 **	2.82	24.06 **	0.95
Gluten index	135.16 **	0.02	63.21 **	6.45	44.00 **	2.43
Ash content	102.38 **	0.03	7.68 *	1.44	6.79 *	1.35
Water absorption	232.96 **	2.14	127.96 **	0.70	30.58 **	0.26
Development time, T1	2.78	28.44 **	214.14 **	1.00	224.03 **	8.44 *
Stability	13.06 *	7.60	5.45	1.98	4.23	1.42
C2	770.06 **	27.56 **	14.44 *	5.06	24.44 **	3.50
C ₁₈	586.51 **	10.51 *	7.87 *	8.18 *	16.78 **	3.51
Falling number	868.97 **	0.86	21.85 **	10.85 *	20.81 **	1.62
Starch damage	2.24	0.31	17.47 **	0.00	14.40 *	0.76
C3	2653.80 **	21.76 **	108.00 **	13.49*	62.60 **	4.55
C4	1263.83 **	0.03	40.29 **	5.98	13.93 *	0.42
C5	2311.20 **	0.04	52.44 **	15.96 *	14.06 *	1.31
Final temp. of gelatinization D3	0.60	11.43 *	1.49	1.20	7.31 *	2.67

Significant at: * $p < 0.05$, ** $p < 0.01$.

Table 4. Quality characteristics of protein complex of flour obtained from the tested cultivars.

Factor	Protein Content (N × 5.7) (%d.m.)	Gluten Content (%)	Gluten Index (%)	Ash Content (% d.m.)	Water Absorption (%)	Development Time, T1 (min)	Stability (min)	C2 (Nm)	C ₁₈ (Nm)
Localization (A)									
Wielichowo	12.3 ^b	31.2 ^b	91 ^a	0.50 ^b	54.4 ^b	3.4	10.0 ^a	0.52 ^a	0.54 ^a
Werbkowice	14.3 ^a	35.4 ^a	82 ^b	0.56 ^a	57.3 ^a	3.5	9.4 ^b	0.40 ^b	0.41 ^b
Fertilization (B)									
N + 0S	13.3	33.4	86	0.53	55.7	3.6 ^a	9.9	0.47 ^a	0.48 ^a
N + 30S	13.4	33.1	86	0.53	56.0	3.3 ^b	9.5	0.45 ^b	0.46 ^b
Cultivar (C)									
Hondia	13.0 ^{cd}	31.8 ^b	90 ^{bc}	0.50 ^b	53.2 ^d	3.4 ^b	10.2	0.48 ^a	0.49 ^a
Pokusa	14.0 ^b	35.9 ^a	84 ^c	0.53 ^{ab}	57.6 ^b	4.5 ^a	9.5	0.46 ^a	0.48 ^{ab}
Belissa	13.5 ^{bc}	36.6 ^a	76 ^d	0.52 ^{ab}	59.0 ^a	4.2 ^a	9.1	0.44 ^b	0.45 ^b
Hybery F ₁	12.7 ^d	29.8 ^b	88 ^{bc}	0.54 ^{ab}	54.0 ^d	3.1 ^b	9.7	0.48 ^a	0.48 ^{ab}
Bonananza	13.4 ^{bc}	32.4 ^b	94 ^a	0.56 ^a	55.4 ^c	2.0 ^c	10.0	0.45 ^{ab}	0.46 ^{ab}

a, b, c, d—values marked with the same letters do not differ significantly at $p < 0.05$ according to Tukey's multiple test.

The addition of sulfur fertilization had no effect on flour protein and gluten content, and neither had any effect on gluten quality, ash content, and water absorption of flours (Table 4). However, we observed lower values of development time and torque in points C2 and C₁₈, which indicates that dough is at risk of becoming less stable and weaker during processing [59]. The differences were statistically significant, but not from a technological point of view. Sulfur application in the research of Wilson et al. [52] caused increased average water absorption of flour, development time, and dough stability determined by farinograph.

Flour obtained from tested wheat cultivars was differentiated in terms of protein properties. Cultivar 'Pokusa' was characterized by the highest baking value—with the highest

protein and gluten content (14.0% and 35.9%, respectively) (Table 4). The lowest baking value was found for cv. ‘Hondia’ and ‘Hybery F₁’, which were also characterized by the lowest water absorption values (53.2 and 54.0%, respectively). Cultivar ‘Belissa’, with one of the greatest protein and gluten contents and the lowest gluten index, was characterized to be the most favorable in terms of water absorption of flour (59.0%) compared to the rest of the cultivars. All tested cultivars showed protein weakening—a dough torque in point C2 below 0.5 Nm, which indicates the appropriate quality to produce bread [60]. High values of dough torque measured 18 min after the mixolab test started (C₁₈) indicate a better tolerance to the weakening of the gluten structure during mixing and gradually increasing the temperature of the dough [60].

The interaction between localization and fertilization was stated only for the dough torque measured 18 min after the test started (C₁₈) (Table 3). The interaction between localization and cultivar was found for protein content, gluten content, gluten index, ash content, water absorption, dough development time, C2, and C₁₈, whereas the interaction between fertilization and cultivar was found only for development time.

Asparagine concentration was positively correlated with factors affecting bread-making quality: gluten content ($r = 0.597$) and water absorption of flour ($r = 0.616$) (Table S3). With the increase of asparagine content in flour, the gluten index decreased ($r = -0.514$), as well as the dough torque in point C₁₈ ($r = -0.952$, respectively). No significant correlation between the asparagine content of wheat and protein or gluten content was found in the research of Malunga et al. [11]. However, weak correlations were found between asparagine content and wheat protein [13], gluten content, gluten index, the Zeleny sedimentation index, and water absorption [56].

The importance of the level of alpha-amylase activity is crucial for the dough fermentation process and for obtaining high-quality bread. Alpha-amylase activity and starch properties of tested flours were characterized by several parameters. The ANOVA results (Tables 3 and S4) indicated that localization had the greatest share in the total variance in the measured quality parameters characterizing the starch complex of flour, such as starch retrogradation C5 (88.8% in the total variance), stability of hot starch paste C4 (84.7%), starch gelatinization C3 (78.2%), and falling number (81.8%) (Table S4). For the wheat samples tested in this study, the cultivar was the second most impactful factor on parameters such as starch damage, contributing to 51.0% of the variance in their concentration.

Tested flours were characterized by average and low alpha-amylase activity, measured as falling number (in the range of 187 to 372 s). Flours obtained from grain cultivated in the Wielichowo localization were characterized by significantly higher falling numbers compared to the Werbkowice localization (Table 5). This lower alpha-amylase activity of grain samples from the Wielichowo localization directly affected the higher values of dough torque measured in points C3, C4, and C5 (2.15, 2.17, and 3.46 Nm, respectively) than from Werbkowice (Table 5).

Additional sulfur fertilization had no negative effect on the starch properties of tested flours. It influenced only the lower value of dough torque in point C3 measures of starch gelatinization and the lower final temperature of gelatinization (1.87 Nm and 74.8 °C, respectively) (Table 5). According to Klikocka et al. [51], sulfur addition had no effect on the starch content of tested wheat cultivars.

Alpha-amylase activity determined by falling numbers varied between the tested cultivars. Cultivar ‘Belissa’ was characterized by the highest value of falling number (on average, 317 s) compared to other cultivars (Table 5). The lowest falling number was stated for cv. ‘Bonanza’ (266 s). Despite the highest falling number value, cv. ‘Belissa’, along with cv. ‘Pokusa’ and cv. ‘Bonanza’, was characterized by the significantly lowest dough torque in points C3 (starch gelatinization), C4 (stability of hot starch paste), and C5 (starch retrogradation) of the mixolab curves. The highest values were stated for cv. ‘Hondia’ and ‘Hybery F₁’.

Table 5. Quality characteristics of starch complex of flour obtained from the tested cultivars.

Factor	Falling Number (s)	Starch Damage (UCD)	C3 (Nm)	C4 (Nm)	C5 (Nm)	Final Temperature of Gelatinization, D3 (°C)
Localization (A)						
Wielichowo	342 ^a	18.2	2.15 ^a	2.17 ^a	3.46 ^a	75.7
Werbkowice	237 ^b	18.8	1.63 ^b	1.16 ^b	1.69 ^b	75.4
Fertilization (B)						
N + 0S	291	18.6	1.92 ^a	1.67	2.58	76.3 ^a
N + 30S	288	18.4	1.87 ^b	1.66	2.57	74.8 ^b
Cultivar (C)						
Hondia	288 ^{bc}	16.3 ^c	2.00 ^a	1.85 ^a	2.84 ^a	75.2
Pokusa	282 ^{bc}	20.3 ^a	1.79 ^b	1.51 ^b	2.36 ^b	74.8
Belissa	317 ^a	19.7 ^{ab}	1.81 ^b	1.62 ^b	2.47 ^b	76.3
Hybery F ₁	296 ^{ab}	17.2 ^{bc}	2.04 ^a	1.90 ^a	2.95 ^a	75.6
Bonanza	266 ^c	18.9 ^{ab}	1.82 ^b	1.46 ^b	2.28 ^b	75.8

a, b, c—values marked with the same letters do not differ significantly at $p < 0.05$ according to Tukey's multiple test.

Starch damage is one of the most important criteria for assessing the quality of baking flour. It affects the water absorption of flour, the rheological properties of dough, the fermentation of the dough, and the structure of the bread crumb [61]. The starch damage of tested flours varied from 14.0 to 21.3 UCD and differed depending on the cultivar (Table 5). The highest average starch damage was found for cv. 'Pokusa', whereas the lowest value was found for cv. 'Hondia'. A wide range of starch damage indicates the possibility of using these flours in the production of many assortments of baking and pastry products. Wheat flours intended to produce bread and confectionery products are characterized by starch damage, usually in the wide range from 14 to 24 UCD [62]. According to Ma et al. [63], an increase in the starch damage of flour significantly decreased the falling number and increased the alpha-amylase activity. One of its benefits is greater susceptibility to amylolytic enzymes, which results in increased maltose and dextrin production during dough fermentation [32,34]. With the increase in the amount of fermentable carbohydrates in the dough, the activity of yeast and bacteria is stimulated [64]. In addition, the increase in the level of starch damage may also favor the increased reduction in carbohydrates, which are also involved in acrylamide formation. The conversion ratio of asparagine to acrylamide could be enhanced when starch damage is increased [34].

Mulla et al. [35] found a correlation between damaged starch content in the flour and acrylamide content in bread. The extent of milling is known to affect the contents by both reducing sugars [65] and amino acids [66]. This observation indicated that the mitigation of acrylamide in bread can be obtained by reducing starch damage in wheat flour [34]. To clearly unravel genotypic differences and their interaction with environmental factors and, especially, nitrogen and sulfur fertilization, further research is needed.

Similar to the research of Malunga et al. [11], no significant correlation was found with asparagine content. Asparagine content was negatively correlated with the falling number ($r = -0.905$) (Table S3). Doughs from flours with higher asparagine content were also characterized by lower consistency in points C3, C4, and C5 ($r = -0.932$, $r = -0.964$, and $r = -0.977$, respectively).

The interaction between localization and fertilization was found for the following quality parameters: falling number and dough torque in points C3 and C5 (Table 3). The interaction between localization and cultivar was found for falling number; starch damage; dough torque in points C3, C4, C5; and final temperature of gelatinization, whereas no interaction between fertilization and cultivar was detected.

3.3. Bread Quality Properties

The ANOVA results (Tables 6 and S5) indicated that the localization had the greatest share in the total variance of the measured acrylamide content in bread (55.8% in the total variance) and the color of the bread crumb (85.5%—parameter a^*). The wheat cultivar had the second most significant impact on parameters such as loaf volume (62.5% of the total variance), acrylamide content (13.5%), and bread crumb color (L 38.7% and b^* 55.2%).

Table 6. F values calculated in the three-way ANOVA made for the qualitative parameters for the bread obtained in the baking trial from the grain of the examined wheat samples.

Parameter	Sources of Variation						
	Localization (A)	S Fertilization (B)	Cultivar (C)	A × B	A × C	B × C	A × B × C
Loaf volume	12.22 **	18.03 **	68.75 **	1.47	20.02 **	1.74	1.51
Bread crumb hardness	119.35 **	25.87 **	4.33 **	3.08	6.25 **	29.19 **	37.75 **
Acrylamide content	1518.09 **	359.17 **	92.03 **	9.21 **	8.15 *	65.03 *	37.92 *
Lcrumb	404.92 **	0.61	85.12 **	1.59	11.63 **	0.84	0.81
a^* crumb	3059.32 **	12.69 **	62.99 **	0.61	25.49 **	4.68 **	12.79 **
b^* crumb	248.16 **	4.36 *	187.01 **	0.00	51.10 **	4.91 **	12.92 **
Lcrust	36.33 **	24.89 **	2.08	0.41	13.96 **	2.03	1.17
a^* crust	0.60	2.85	4.03 *	6.67 *	9.71 **	1.43	0.40
b^* crust	39.13 **	33.20 **	3.48 *	0.03	7.25 **	1.96	2.14

Significant at: * $p < 0.05$, ** $p < 0.01$.

The data on bread's physical characteristics are shown in Table 7. Bread obtained in laboratory baking was characterized by a proper appearance with the shape of a well-risen loaf and the right color of the crust, as well as a relatively uniform porosity of the crumb (Figures 1–4). The most favorable in terms of baking value and the possibility of using it for baking purposes was bread obtained from grain cultivated in Wielichowo with the highest bread volume (322 cm³) and the lowest acrylamide content (11.9 µg 100^{−1} g) compared to samples from Werbkowice (Table 7).

Table 7. Quality characteristics of bread obtained in laboratory baking from tested cultivars.

Factor	Loaf Volume (cm ³ /100 g)	Bread Crumb Hardness (N)	Acrylamide Content (μg 100 ^{−1} g)	Breadcrumb Color			Bread Crust Color		
				<i>L</i>	<i>a</i> *	<i>b</i> *	<i>L</i>	<i>a</i> *	<i>b</i> *
Localization (A)									
Wielichowo	322 ^a	14.9 ^a	11.9 ^b	74.2 ^a	−1.07 ^b	18.44 ^a	53.36 ^a	13.97	30.45 ^a
Werbkowice	315 ^b	11.9 ^b	17.9 ^a	70.1 ^b	−0.23 ^a	17.60 ^b	49.73 ^b	13.83	27.88 ^b
Fertilization (B)									
N + 0S	323 ^a	12.7 ^b	16.4 ^a	72.2	−0.68 ^b	17.97 ^b	50.04 ^b	14.06	27.98 ^a
N + 30S	315 ^b	14.1 ^a	13.3 ^b	72.1	−0.62 ^a	18.08 ^a	53.04 ^a	13.70	30.35 ^a
Cultivar (C)									
Hondia	334 ^a	12.7 ^b	14.1 ^b	75.1 ^a	−0.58 ^b	17.25 ^c	52.34	14.23 ^a	30.02 ^{ab}
Pokusa	303 ^c	13.8 ^{ab}	14.2 ^b	69.2 ^d	−0.45 ^a	18.26 ^b	50.35	14.28 ^a	28.47 ^{ab}
Belissa	297 ^c	14.3 ^a	17.8 ^a	72.7 ^b	−0.72 ^c	18.81 ^a	51.64	13.95 ^{ab}	28.97 ^{ab}
Hybery F ₁	337 ^a	12.9 ^b	13.9 ^b	72.3 ^{bc}	−0.75 ^c	17.08 ^c	52.60	13.24 ^c	30.10 ^a
Bonanza	323 ^b	13.5 ^{ab}	14.4 ^b	71.5 ^c	−0.75 ^c	18.72 ^a	50.78	13.81 ^{ab}	28.27 ^b

L , lightness, ($L = 0$ is black; $L = 100$ is white); a^* , green-red opponent colors ($-a$ = green; $+a$ = red); b^* , blue-yellow opponent colors ($-b$ = blue; $+b$ = yellow). a, b, c, d—values marked with the same letters do not differ significantly at $p < 0.05$ according to Tukey's multiple test.

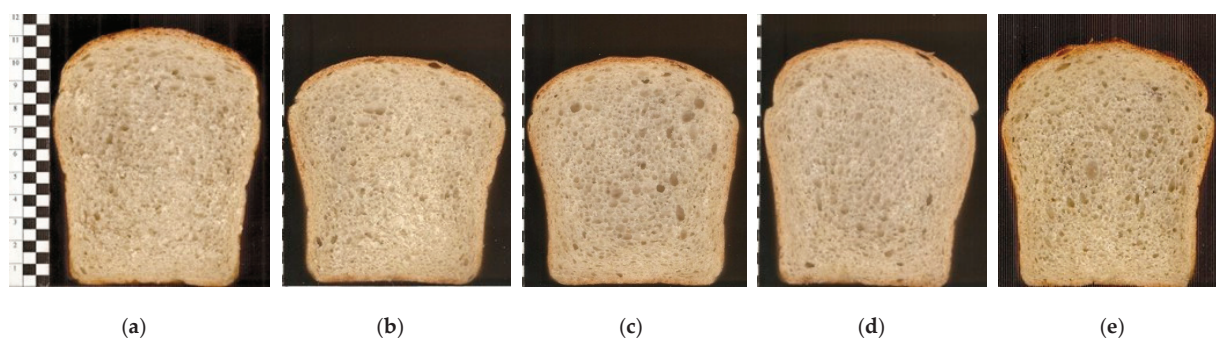


Figure 1. Cross-sectional view of bread from the tested wheat cultivars from Wielichowo localization with N + 0S fertilization: (a) Hondia; (b) Pokusa; (c) Belissa; (d) Hybery F₁; (e) Bonanza.

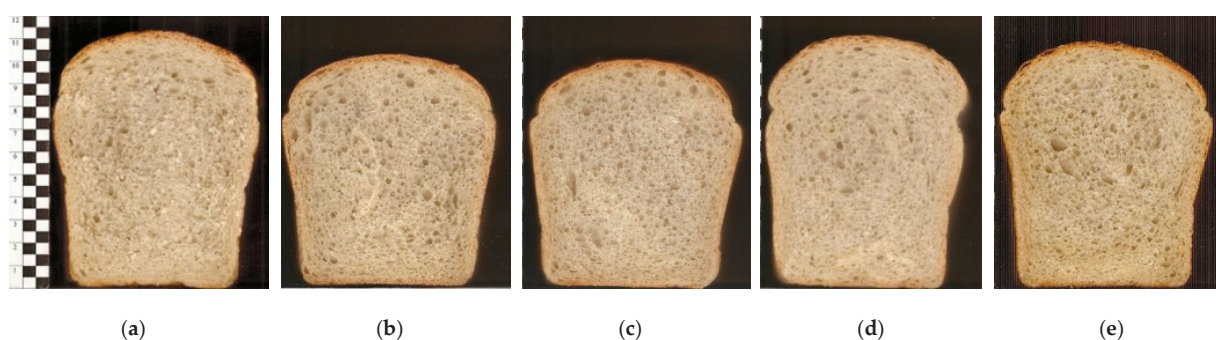


Figure 2. Cross-sectional view of bread from the tested wheat cultivars from Wielichowo localization with N + 30S fertilization: (a) Hondia; (b) Pokusa; (c) Belissa; (d) Hybery F₁; (e) Bonanza.

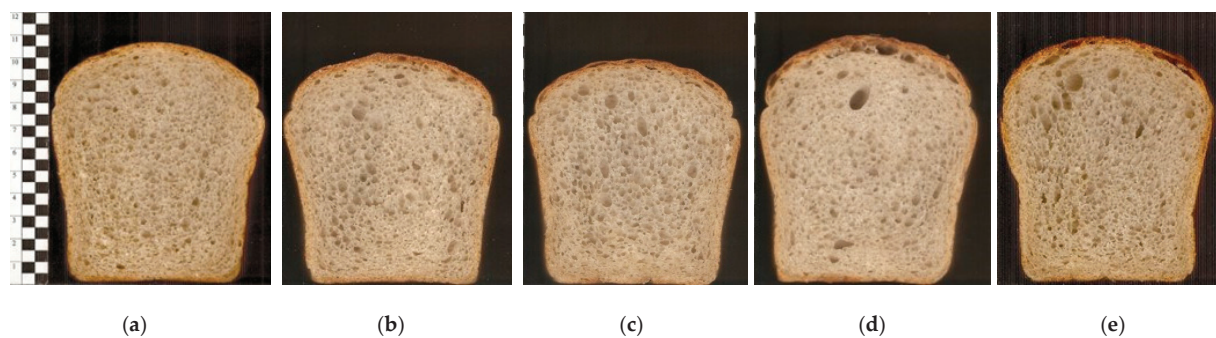


Figure 3. Cross-sectional view of bread from the tested wheat cultivars from Werbkowice localization with N + 0S fertilization: (a) Hondia; (b) Pokusa; (c) Belissa; (d) Hybery F₁; (e) Bonanza.

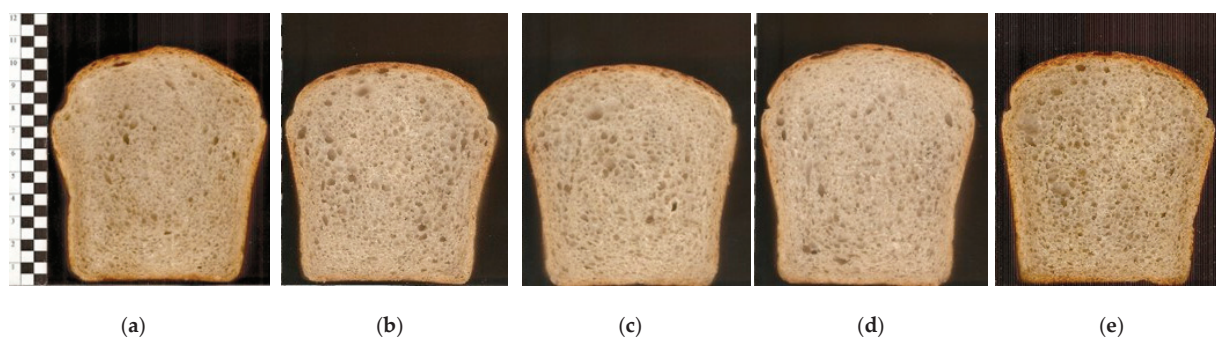


Figure 4. Cross-sectional view of bread from the tested wheat cultivars from Werbkowice localization with N + 30S fertilization: (a) Hondia; (b) Pokusa; (c) Belissa; (d) Hybery F₁; (e) Bonanza.

The addition of sulfur fertilization had a negative effect on the bread volume by lowering it from 323 cm³ to 315 cm³. Additionally, the hardness of the bread crumb was increased to 14.1 N (Table 7). Shahsavani and Gholami [67] and Li et al. [68] found that wheat grown at an intermediate sulfur fertilization rate yielded the highest loaf volume. Asparagine reductions achieved by increasing sulfur fertilization partially coincided with a loss in the functionality of bread.

An undoubtedly positive health effect of sulfur fertilization was lowering the acrylamide content in bread, on average, from 16.4 to 13.3 µg 100⁻¹ g (Table 7). Considering the results of individual samples in a given location, the highest reduction in acrylamide content was found for grain samples cultivated in Wielichowo (on average, 3.6 µg 100⁻¹ g lower) than in Werbkowice (on average, 2.5 µg 100⁻¹ g lower) (Table S2). Bread obtained from cv. 'Hondia' and 'Belissa' cultivated with sulfur fertilization was characterized by the greatest reduction in acrylamide content in both localizations (on average, 3.7 and 6.9 µg 100⁻¹ g lower).

Unlike the results of Claus et al. [7], bread obtained from wheat cultivars belongs to Class A—good category wheat (cv. 'Hondia', 'Pokusa')—and shows a lower value of acrylamide than from other bread category cultivars ('Belissa', Hybery F₁', 'Bonanza'). It is possible to indicate cultivars with good baking value, the bread obtained from which will be characterized by a low acrylamide content.

Among tested cultivars, cv. 'Hondia' and cv. 'Hybery F₁' with the lowest starch damage of flour were characterized by the highest bread volume, the lowest bread crumb hardness, and one of the lowest acrylamide contents. Cultivar 'Belissa', with the second highest degree of starch damage, was characterized significantly by the lowest bread volume with the highest bread crumb hardness and the highest acrylamide content (296.9 cm³, 14.3 N and 17.8 µg 100⁻¹ g, respectively), and was rated as the least favorable.

Browning products obtained from the Maillard reaction influence sensory perception. Bread crust obtained from flours with higher protein and amino acid contents was darker. Localization and fertilization, but not cultivar, were factors that significantly influenced the lightness *L* of the tested bread crusts (Table 7). The highest value of brightness *L* of the crust was obtained for the bread samples obtained from Wielichowo (with also a lower value of protein content, asparagine content in grain, and acrylamide content in bread), and with N + 30S treatment (with lower acrylamide content) (Table 7). Li et al. [68] indicated that the environmental factor and cultivar, but not sulfur fertilization, influence the brightness of the bread crust. Bread obtained from grain cultivated with sulfur was also characterized by a smaller share of green and a greater share of red in the color of the crumb, higher brightness of the crust, and a higher share of red tone.

Asparagine content influenced the acrylamide content in bread ($r = 0.768$) (Table S3). Bread from flours with higher asparagine content was also characterized by darker crumb color and a greater share of red color (a^* crumb) ($r = -0.733$ and $r = 0.897$, respectively). In this work, we did not observe the influence of starch damage on the acrylamide content in bread when the results of all cultivars, fertilization, and location were compared. However, bread obtained from flour with higher starch damage was characterized by darker crust color, with a greater share of red, and a smaller share of yellow (correlation with L^* value $r = -0.607$, $a^* r = 0.548$, and $b^* r = -0.545$, respectively).

The results of a principal component analysis (PCA) demonstrated that the first two principal components (PC1 and PC2) accounted for 8.3% of the variation (Figure 5). PC1 explained 51.4% and PC2 explained 30.9% of the variation. Figure 5 shows that cultivars 'Hondia' and 'Hybery F₁' with the lowest asparagine content, starch damage, water absorption of flour, bread crumb hardness, acrylamide content, and the highest loaf volume belong to one group, while the remaining cultivars form three separate groups. Comparing the position of the cases on the chart with the forms of components and factor loadings, it can be concluded that the cv. 'Hondia' is negatively correlated with PC1 and positively with PC2, while the cv. 'Hybery F₁' is negatively correlated with PC1 and

PC2. However, the cultivars ‘Bonanza’, ‘Belissa’, and ‘Pokusa’ were positively correlated with PC1.

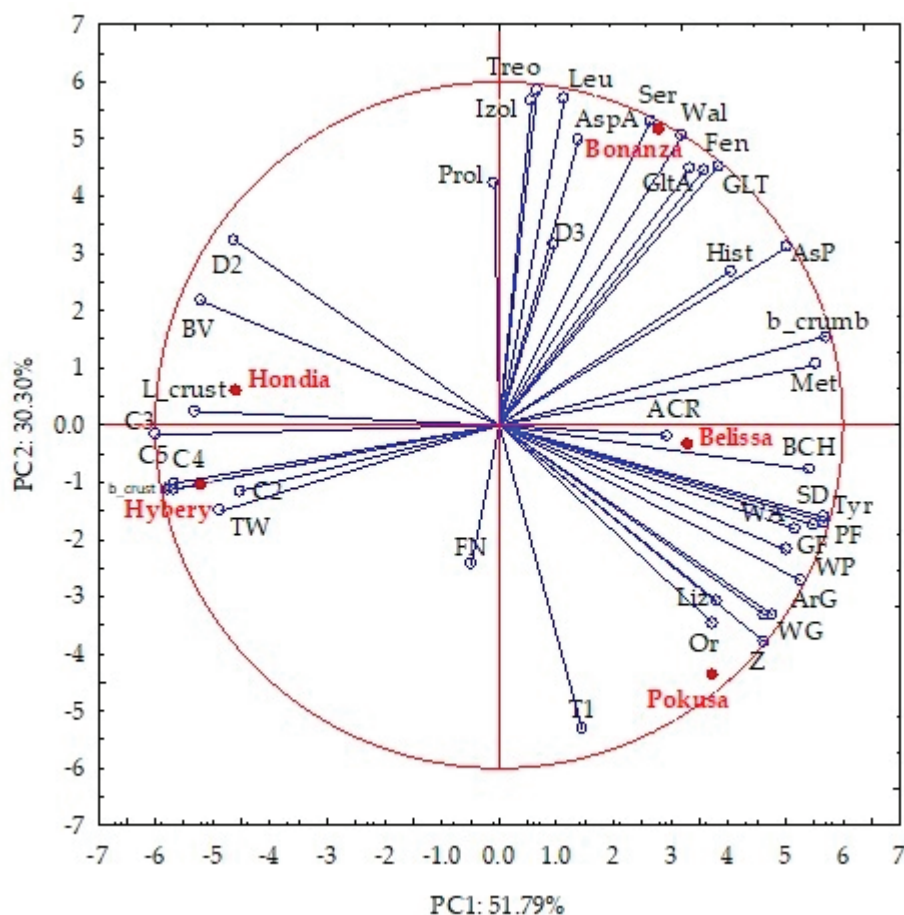


Figure 5. Variable graph of PCA showing the quality parameters and cultivars of wheat. Explanations: TW—test weight, FN—falling number, Z—Zeleny index, WP—wheat protein content, PF—flour protein content, WG—wheat gluten content, SD—starch damage, WA—water absorption, T1—development time, BV—bread volume, BCH—bread crumb hardness, ACR—acrylamide content, Treo—threonine, Leu—leucine, Ser—, Wal—valine, Fen—phenylalanine, Izol—iso-leucine, Prol—proline, GltA—glutamic acid, GLT—glutamine, Hist—histidine, AsP—asparagine, AspA—aspartic acid, Met—methionine, Tyr—tyrosine, ArG—arginine, Liz—lysine, Or—ornithine.

Figure 5 shows that cultivars ‘Hondia’ and ‘Hybery F₁’ are classified in the left section of the chart, which indicates relatively higher values of bread volume (BV), brightness of crust (Lcrust), starch gelatinization (C3), amylolytic activity (C4), retrogradation (C5), and protein weakening (C2), while featuring low values of amino acids, total protein, and gluten content. However, cultivars ‘Bonanza’, ‘Belissa’, and ‘Pokusa’ scored low values of parameters such as the starch–amylolytic complex and higher amino acid values than the previously mentioned cultivars. The cv. ‘Bonanza’ is characterized by the highest value of aspartic acid, threonine, leucine, serine, and valine among the three cultivars mentioned. The cv. ‘Pokusa’ is characterized by the highest protein content both in grain and flour regarding gluten content and arginine, and the most favorable features characterizing the quality of gluten proteins, i.e., the Zeleny sedimentation index and water absorption of flour. The obtained results indicate a positive correlation of the starch damage (SD) with water absorption (WA) and bread crumb hardness (BCH), and a negative correlation with the brightness of a crust (Lcrust) and starch retrogradation (C5). Cultivars ‘Belissa’, ‘Bonanza’, and ‘Pokusa’ were characterized by higher average values of starch damage (SD), water absorption (WA), and bread crumb hardness (BCH). Bread obtained from

cultivars ‘Hondia’ and ‘Hybery F₁’ had the lowest acrylamide content and bread from cultivars ‘Bonanza’, ‘Belissa’ and, ‘Pokusa’ had the highest acrylamide content due to the high protein content and amino acid content, including asparagine and aspartic acid, and higher access to maltose and dextrin products during fermentation as a result of high starch damage.

4. Conclusions

To our knowledge, this work presents the first comprehensive analysis related to the accumulation of asparagine in grain and acrylamide content in bread, including the assessment of the baking value of five winter wheat varieties cultivated in Poland.

Our research showed a significant influence of localization (environment) and cultivar and their interaction on the asparagine and acrylamide content and technological parameters of grain. The influence of sulfur application on the asparagine content in grain and on the baking value has not been confirmed. However, we found a negative effect of sulfur fertilization on the acrylamide content in bread. The obtained results indicate that, on the one hand, there is no effect of sulfur on the asparagine content in grain and, on the other hand, a decrease in acrylamide in bread may be surprising because the highly significant positive correlation between these features was confirmed in our research. Probably, the effectiveness of sulfur in reducing the asparagine in the endosperm depends on the wheat cultivar and the location of the crop, which requires further research and confirmation.

The food industry requires cultivars with reduced free asparagine content and favorable baking properties, and which show stable characteristics regardless of weather conditions. The obtained localization × cultivar interactions indicate different responses of wheat cultivars depending on habitat conditions. The interaction was confirmed in the asparagine and acrylamide content and bread-making quality. In our research, the asparagine content in tested cultivars ranged from 235 to 588 mg kg⁻¹, the acrylamide content from 8.0 to 23.4 µg 100⁻¹ g, and the protein content in wheat grain from 12.3 to 17.6% d.m. (Table S2). Considering the quality requirements of the baking industry (high protein content) and food safety requirements (low asparagine and acrylamide content), the most valuable cultivar is ‘Pokusa’. Cv. ‘Pokusa’ is characterized by high protein content in grain (15.8% d.m.) and one of the lowest asparagine contents in grain (397 mg kg⁻¹) and acrylamide content in bread (14.2 µg 100⁻¹ g). From the point of view of the health of the raw material, we can also recommend the ‘Hybery F₁’ cultivar. Bread obtained from this cultivar has the lowest acrylamide content (13.9 µg 100⁻¹ g), but the grain is characterized by the lowest protein content (14.6% d.m.) and gluten content (29.7%) among tested cultivars.

A novel aspect of our research is the analysis of the influence of the amylolytic complex and starch damage on the acrylamide content in bread. Our results indicate a significant negative correlation of starch quality parameters with asparagine and acrylamide content. The results also indicate differences in cultivars. Among others, cv. ‘Belissa’, with the highest falling number and starch damage values, is characterized by a higher asparagine content in grain and acrylamide content in bread. Further research should therefore consider the aspect of starch properties on the formation of asparagine in grain and acrylamide in bread.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture14020207/s1>. Table S1: The ratio of the factorial SS to the total sum of squares illustrating the percentage share of the total variance for the qualitative parameters of grain of the examined wheat samples. Table S2: Protein and asparagine content in wheat, starch damage of flour, and acrylamide content of bread depend on cultivar, localization, and fertilization. Table S3: Correlation between asparagine concentration, acrylamide content, and quality parameters. Table S4: The ratio of the factorial SS to the total sum of squares illustrating the percentage share of the total variance for the qualitative parameters of the flour obtained from the grain of the examined wheat samples. Table S5: The ratio of the factorial SS to the total sum of

squares illustrating the percentage share of the total variance for the qualitative parameters for the bread obtained in the baking trial.

Author Contributions: Conceptualization, A.S. and G.P.; methodology, A.S., G.P. and M.R.; validation, A.S. and O.Ś.; formal analysis A.S.; investigation, A.S., E.A. and O.Ś.; resources, A.S. and M.R.; data curation, A.S. and D.K.; writing—original draft preparation, A.S.; writing—review and editing, A.P.-C. and M.R.; visualization, A.S.; supervision, A.S.; project administration, A.S.; funding acquisition, A.S. and G.P. All authors have read and agreed to the published version of the manuscript.

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Article

Impact of Biochar Dose and Origin on Winter Wheat Grain Quality and Quantity

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Abstract: The agricultural application of biocarbons (biochar) derived from different biomass sources in the process of pyrolysis is a promising solution for crop productivity and quality, soil health improvement, and carbon sequestration. In a three-year study, the effects of low doses of biochar (1 t·ha⁻¹ and 3 t·ha⁻¹) of different origins on winter wheat grain quantity and quality were tested. Six different biochar types were used: biochar derived from wheat husk (WHB), (2) extracted medical plant biomass biochar (MPB), (3) wood chip biochar (WCB), (4) wood sawdust biochar (SB), (5) biochar made from straw of rye (RSB), and (6) meat and bone biochar (MBMB). Higher doses of biocarbon had a positive effect only on wet gluten content. The use of different types of biochar showed a significant impact on grain parameters; however, the results were different in different years of this study. Among the tested biochars, SB (Saw Dust biochar) showed rather good results for most of the parameters tested (the highest grain yield in 2018, the highest weight of 1000 g in 2019, the lowest wet gluten content and gluten index in 2020, the lowest falling number in 2019, and the highest Zellenys index in 2019). MBMB biochar was one of the highest yielders in 2018, had the highest wet gluten content in 2018, and the highest gluten index in 2019 and 2020; the lowest Zelleny's sedimentation index in 2019; and one of the lowest in 2020. Those made SB and MBMB the most promising biochars tested in this study.

Keywords: biochar; winter wheat; cereal yields; grain quality

1. Introduction

The recent increase in the world's population means that the demand for food is also increasing. This demand, depending on the scenario, will have to increase by 35% to 56% between 2010 and 2050 [1]. Some estimates indicate that food production between 2005 and 2050 will have to increase even more, even up to 70% by 2050 [2]. The total earth's population is expected to reach 9.7 billion people in 2050 [3]. These projections are becoming realistic through the breeding of more productive and higher-yielding varieties, but also through agrotechnical advances and more efficient use of nutrients [4,5]. Another way to meet those growing demands is the introduction of products and substances that improve soil fertility and its properties, and thus soil productivity. One of the key aspects is defining soil properties: biological, physical, chemical, and microbiological. Thanks to such determinations, we can observe how agricultural activities affect the soil environment and ecosystem services provided by soil [6,7]. The impact of crop cultivation under intensive agriculture principles is rarely positive. Recently, one of the main issues and challenges of global agriculture has been coping with negative environmental impacts, such as the loss of soil organic matter (SOM) [8,9]. SOM is of unique importance for many biochemical processes, including climate change mitigation potential and agricultural water management. This is prompting a search for alternative sources of organic matter that can contribute to soil organic matter and humus concentration and thus improve soil fertility [10]. Significant changes in the quality and quantity of humus compounds can

be caused by the addition of an external source of organic matter [11]. The challenge of increased sequestration of carbon in soil is also a challenge for the European Union's (EU) policymakers, as reflected in recent programs and strategies, of which the European Green Deal [12] is of the greatest importance. In the European Union (EU), current legislation allows the legal use of biocarbon as a soil improver for agricultural purposes, including organic farming, according to specific criteria for contaminant content, including heavy metals and Polycyclic aromatic hydrocarbons (PAHs) [13].

One of the external sources of organic matter for soil is biochar (also known as biocarbon), which has been one of the leading research topics in Europe in recent years. Industries such as biocarbon generation, its modifications, and the manufacture of biocarbon-based products are developing rapidly. The International Biochar Initiative defines biocarbon as a fine-grained carbonate with a high organic carbon content and low degradability, obtained by pyrolysis of biomass and biodegradable wastes [14].

Biocarbon is primarily used as a measure in environmental protection and agriculture. It is a solid renewable fuel extracted from various types of biomass by pyrolysis [15]. The material from which biocarbon is produced can be diverse, and almost any biomass can be the substrate for biocarbon generation (energy crops, forestry waste, rapeseed straw, sunflowers, maize cobs, but also sewage sludge, organic waste, or manure) [16–20]. Biochar can enhance soil fertility by providing a habitat for microorganisms and beneficial bacteria. It acts as a porous structure, offering a refuge for soil microbes and promoting their activities. This microbial activity helps in nutrient cycling and improves the availability of essential nutrients to plants. Biochar has a high cation exchange capacity (CEC), meaning it can retain and exchange essential nutrients such as nitrogen, phosphorus, and potassium. This can lead to better nutrient availability for plant uptake and reduce nutrient leaching, promoting more efficient fertilizer use [21]. Moreover, biochar's porous structure also improves water retention in soils. It can hold onto water during wet periods and release it during dry periods, helping to maintain more consistent moisture levels. Additionally, it improves drainage in clayey soils, preventing waterlogging and enhancing aeration. The water-holding potential of biochar is especially visible on coarse-textured soils [22]. Biochar can also help regulate and stabilize soil pH. It has a neutral to slightly alkaline pH, and when added to acidic soils, it can contribute to raising the pH, making the soil more suitable for a broader range of crops, and, again, contributing to nutrient availability for plants [23]. Some studies suggest that biochar can bind with heavy metals in the soil, reducing their availability for uptake by plants. This may be beneficial for grain quality by minimizing the accumulation of heavy metals in grains [24–26].

The above-mentioned properties of biocarbon indicate the great potential for the use of this material in cereal crop production. Among cereals in the European Union, wheat accounts for nearly 46% of grain production. It is also an important crop in Poland, as it takes first place in terms of its share in the sowing structure (28%). In the case of wheat, it is not only the volume of grain yield obtained that is important, but also its quality, which is determined by the requirements of the milling and baking industry [27].

The impact of biochar (biocarbon) on wheat yield can depend on various factors mentioned above, including the type of biochar used (determining the size of carbon particles and its chemical composition) and its application rate as main factors. The aim of this study was to determine the effect of the type (biochar origin) and dose of biocarbon on winter wheat yields and selected characteristics of the technological value of grain and flour.

2. Materials and Methods

In the three growing seasons of 2017/2018, 2018/2019, and 2019/2020, a two-factor pot experiment was conducted in six replicates at the Vegetation Experiment Hall of IUNG-PIB in Puławy, Poland. The first factor of the first order was the type of biochar (source material that was used during the pyrolysis process): (0) control object (no biochar added), (1) wheat husk biochar (WHB), (2) biochar made from extracted medical plant biomass

(MPB), (3) wood chip biochar (WCB), (4) wood sawdust biochar (SB), (5) biochar made from rye straw (RSB), and (6) meat and bone biochar (MBMB). WHB and MPB biocarbons were market products with the appropriate certificates (certifying their quality and composition) purchased in Germany. WCB biocarbon was a market product purchased in Poland (it was available on the market at the time but is now discontinued). The other three biocarbons (SB, RSB, and MBMB) were produced for the purposes of this experiment by an external entity. Pyrolysis temperature and time, as well as the chemical composition of the tested biocarbons, are given in Table 1. Subjectively, according to this study authors, MBMB biocarbon was the most fine (smallest particle size). In contrast, the RSB biocarbon was characterized by the largest particles (fragments of charred stalks—straws were visible). Other biocarbons tested had medium particle sizes. Charred seed husks were visible in WHA biocarbon, while pieces of charred plants have been visible in MPB biocarbon. Accurate particle size measurements were not made under this study. The second factor of the experiment was the rate (dose) of biochar: (1) 1 t·ha^{−1}, (2) 3 t·ha^{−1}. The experiment was conducted in a completely randomized design, in Micherlich pots filled with 7 kg of soil. Soil was taken from a field where the crop rotation was 100% cereals. The soil for this study was taken from the top layer (0–30 cm) of Haplic Luvisol, made of clay. The content of selected elements in the soil was: total carbon—0.90%; organic carbon—0.78%; total N—0.10%; P₂O₅—27.7 mg·100 g^{−1}; K₂O—28.2 mg·100 g^{−1}; pH—6.08. The soil for the experiments was taken after 3 years of research and stored frozen. The soil was replaced in each year of this study, and fertilizers and biocarbons were added in the same way in each year of this study. Thus, each year, the seeds were sown in new soil with the same physical and chemical parameters. Because of this design, it was impossible to track the addition of carbon over time, and the results of each year must be evaluated independently. The experimental plant was winter wheat, variety Hondia (10 plants per vase). Before filling the vases with soil, the appropriate type of biocarbon was added to the substrate along with the relevant fertilizers and then mixed with the soil. Nitrogen in the form of NH₄NO₃ was applied at a dose of 3.6 g N·vase^{−1}: 1/2 at the beginning of vegetation (spring) and 1/2 dose at the BBCH 30–32 (*ger.* Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie)—a stem shooting stage of wheat. Fertilization of the remaining components per pot was as follows: P₂O₅—2.52 g in the form of KH₂PO₄; K₂O—2.04 g in the form of K₂SO₄; Mg—0.5 g in the form of MgSO₄. In addition, iron (50 mg per pot), boron (5 mg per pot), manganese (3 mg per pot), and copper (3 mg per pot) were also added to the substrate in the forms of Fe(NH₄)₂, C₃H₆N₆·x·BH₃O₃, C₁₀H₁₂MnN₂O₈·2Na, and Cu(NH₄)₂, respectively. The moisture content of the substrate was maintained at 60% water capacity throughout the growing season. The characteristics of the biocarbon types are shown in Table 1.

Table 1. Chemical composition of the tested biochar.

Type of Biochar	Total Carbon (%)	N (%)	S (%)	P (%)	pH	Pyrolysis Temperature (°C)	Time of Pyrolysis (min)
WHB	70.8	3.9	0.074	0.320	8.50	550	nd ¹
MPB	63.5	1.8	0.890	0.095	10.00	600	nd
WCB	67.6	2.4	<0.100	0.330	6.79	500	4–7
SB	42.6	3.6	0.224	4.420	6.78	550	240
RSB	70.5	1.2	0.146	0.260	9.65	550	180
MBMB	75.6	1.9	0.136	0.120	8.32	550	240

¹ nd—no data (the producer did not provide it in the certificate).

When wheat reached the full maturity-growing stage, tested plants were harvested. After the harvest, the following were determined: grain yield per pot and selected characteristics of the technological value of grain and flour: weight of 1000 grains (PN-68/R-74017 [28]), falling number (PN-EN ISO 3093 [29]), wet gluten content (PN-A-74042 [30]), gluten index, and Zelleny sedimentation index (PN-EN ISO 5529 [31]).

The falling number was determined using the Hagberg-Perten method [29]. The grain sample was ground on a laboratory mill (grinder). An automatic equipment *Falling Number 1500* was used for the determination. A total of 7 g of flour with a moisture content of 15% was weighed for analysis in order to ensure a constant level of dry matter content. A sample of middlings in a viscometric tube, together with a stirrer, was placed in a boiling water bath. The use of equipment automatically measured the stirrer's rate of descent in the starch glue. The determination was performed in duplicate. The amount of wet gluten in wheat grain and its index were determined.

The amount of wet gluten in wheat grain and its index were determined according to PN-A-74042 [30]. Grain samples were ground on a laboratory mill (grinder). A *Gluten Index System* was used to determine the amount of wet gluten and its quality. The method involves kneading the dough (with 10 g of flour sample and 4.8 cm³ of 2% NaCl) and washing out the starch with a 2% NaCl solution. The process was carried out automatically in a single sequence for 5 min. Centrifugation of the formed gluten was carried out on sieve cassettes at 6000 $\pm$ 5 rpm.

The Zeleny sedimentation index was determined according to PN-EN ISO 5529 [31] for wheat. The determination of sedimentation index and Zeleny Test was carried out on an apparatus consisting of a measuring panel and a SWD—89 Sadkiewicz type shaker (Sadkiewicz Instruments, Bydgoszcz, Poland).

Average monthly temperatures during subsequent years of this study are given in Table 2.

Table 2. Monthly temperatures during the subsequent year of the experiment and multi-year annual (1981–2010) (°C).

Month	2017/2018	2018/2019	2019/2020	1981–2010
IX	14.1	15.5	14.4	13.3
X	9.5	10.0	10.8	8.0
XI	4.5	4.2	6.4	2.7
XII	2.4	0.9	3.1	−1.4
I	0.6	−2.4	1.7	−3.3
II	−3.5	2.9	3.4	−2.3
III	0.3	5.7	4.7	1.6
IV	13.9	10.0	8.9	8.7
V	17.7	13.9	11.9	14.5
VI	19.6	22.7	19.1	17.2
VII	21.0	19.4	19.3	19.5
VIII	21.1	20.4	20.3	17.8

The results were statistically analyzed with the Tukey test at $p \leq 0.05$.

3. Results and Discussion

3.1. Winter Wheat Yields

Winter wheat grain yield did not depend on the biocarbon dose (Figure 1). However, in the first year of this study, the type of biocarbon had a significant effect on winter wheat grain yield. Both biocarbons from forestry waste (WCB and SB) had a positive effect on yields (Figure 2). It should also be noted that in the other years of this study, the differences were not statistically significant, but the introduction of biocarbon into the soil resulted in a trend towards higher grain yields in winter wheat (Figure 2), regardless of the type of biocarbon. On the other hand, recent studies showed that different types (origins) of biochar can have a significant effect on yields. For example, Lilli et al. [32] have proven that tomato yields can increase even up to 175% when treated with biochar of specific origin and with appropriate fertilization. A study by Gebremedhin et al. [33] indicates a beneficial effect of biocarbons on wheat yield and yield structure traits. The application of biocarbon at 4 t·ha^{−1} resulted in an increase in grain and straw yield compared to the control. Różyło et al. [24] found that amendment of sewage sludge with 5% biochar

can significantly improve yield quantity, but also some grain quality traits. In a study by Khan et al. [34], an increase in wheat grain yield was also observed, but after the application of biocarbon at much higher doses than in the present study ($20 \text{ t} \cdot \text{ha}^{-1}$ and urea at $150 \text{ kg N} \cdot \text{ha}^{-1}$). A positive effect of biochar amendments on cereal yields, especially when combined with nitrogen fertilization, has been reported in multiple studies [35–38]. Zhu et al. [39] also found that biochar amendment to soil could also stimulate the growth of crop root systems, which can also be a factor that, over time, improves the utilization rate of nutrients available in soil.

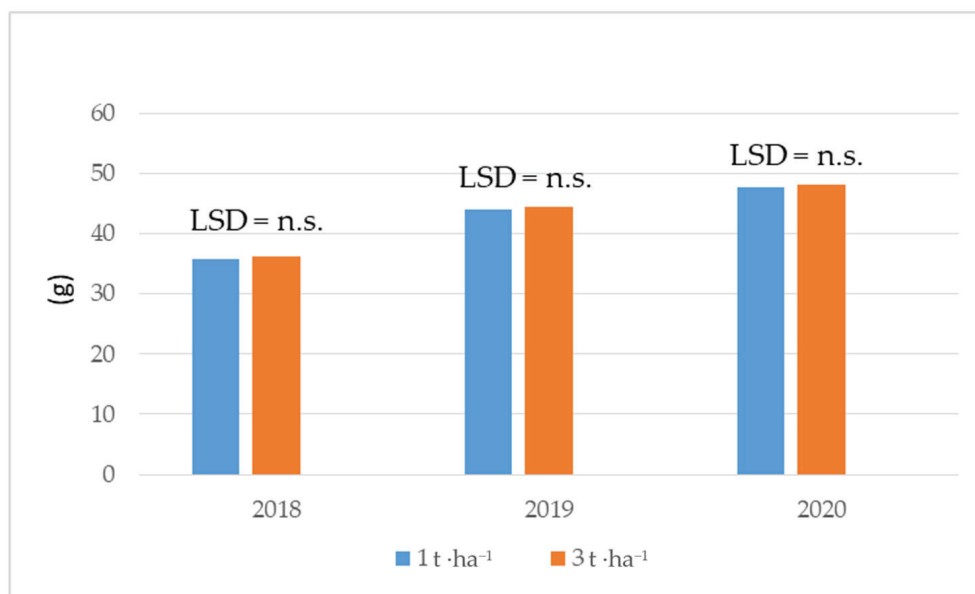


Figure 1. Winter wheat grain yield (g per pot) depends on the biochar rate (g per pot).

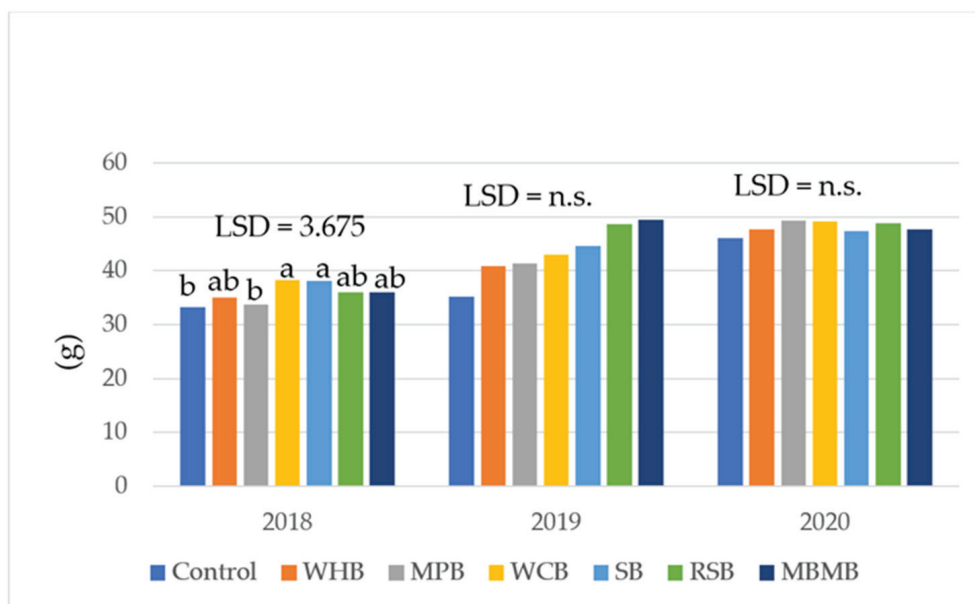


Figure 2. Winter wheat grain yield (g per pot) in the following years of the experiment, depending on the biochar type. Different lowercase letters indicate statistically significant differences between biochar types.

No statistical differences between tested biochar (and control) were found in the third year of this study, and all tested objects reached high yields (of about 45–50 g per pot). The yields for almost all objects (also for control) showed a trend to be the highest, and

at a similar level, in the third year of this study. This may indicate that in the third year of this study, factors other than biochar amendment were mainly responsible for wheat yields. As the soil used in the experiment was removed after each year of the experiment, the increasing saturation of the soil sorption complex with nutrients in the following years could not be a factor. The possible answer is that biochar impact on plant yields was strongly affected by natural conditions, which were, naturally, slightly different in each year of this study.

3.2. Grain Characteristics

The grain of wheat utilized for consumption must be distinguished by its quality, which is determined by grain and flour quality parameters. In general, a distinction is made between the physical parameters of the grain (e.g., 1000 grain weight) and the qualitative parameters (e.g., falling number, Zeleny sedimentation index value, gluten content, gluten index). In our study, the assessment concerned selected physical and qualitative properties of the grain.

3.2.1. Mass of Thousand Grains

The mass of a thousand grains is a measure used in agriculture to assess the weight of a specific number of grains. It is an important parameter as it provides information about the size and weight of individual grains. It can be influenced by crop genotype, growing conditions, and agricultural practices. Farmers and researchers use this information to evaluate crop yield and seed quality and to make decisions about seed selection and planting practices.

In all years of this study, only a trend towards higher 1000 grain weight was found with the application of biocarbon at $3 \text{ t} \cdot \text{ha}^{-1}$. However, the differences were not statistically proven (Figure 3). Billah et al. [40] found that an amendment of biochar can significantly improve the mass of a thousand grains. However, the strength of the effect is supported by the size of the biochar particles—the smaller the particles; the more visible the positive effect.

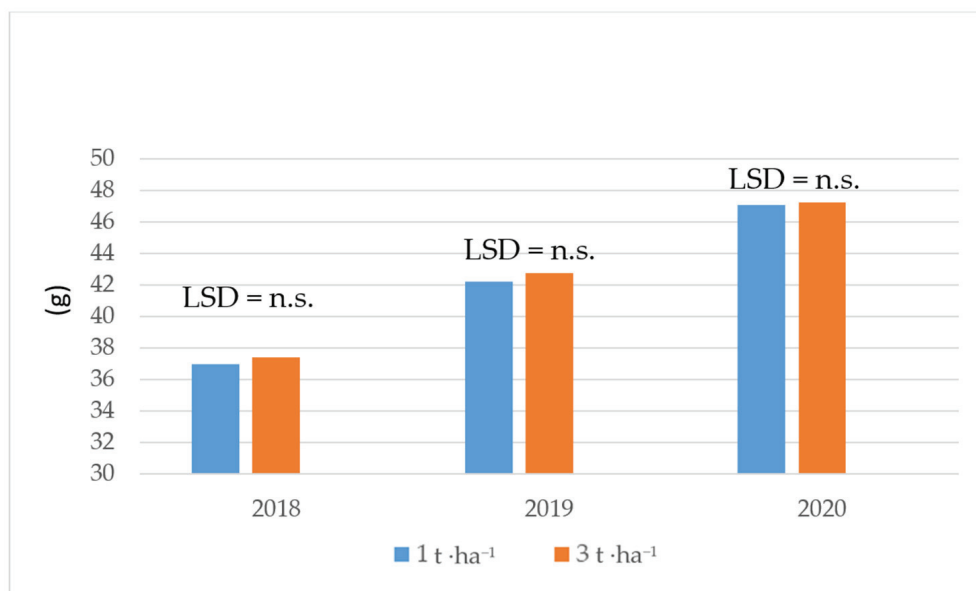


Figure 3. Winter wheat weight of 1000 grains (g) in the following years of the experiment, depending on the biochar dose.

Analysis of the effect of the type of biocarbon on the weight of 1000 grains showed that only in the second year of this study, amendments of biocarbon significantly increased the mass of thousand grains compared to the control object (no added biocarbon). However,

none of the tested biochar types showed statistically better results than others in this matter. Amendment of biocarbon obtained from woody biomass (WCB and SB) again showed a trend to have the best potential of 1000 grain mass (similarly as for yield potential) (Figure 4).

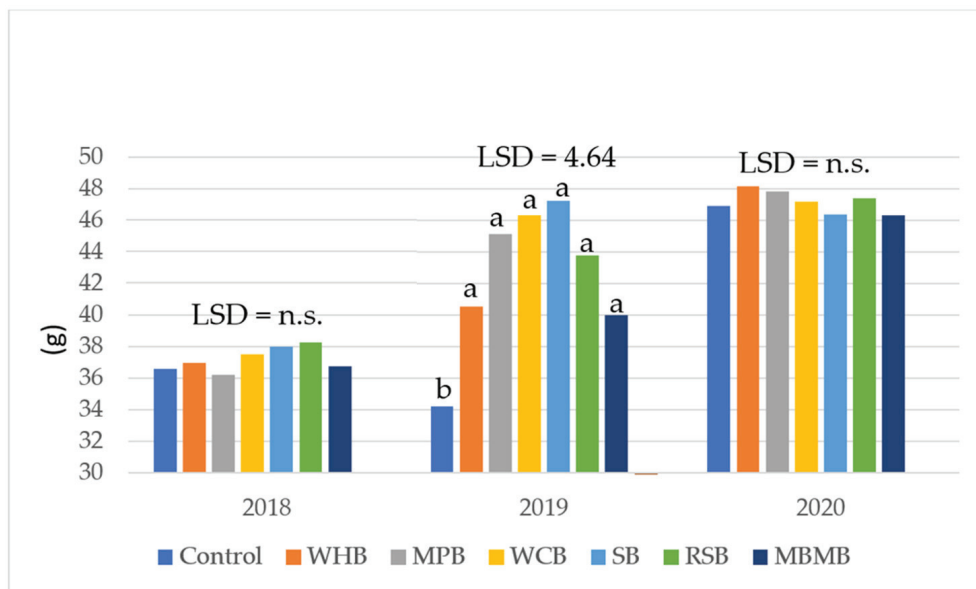


Figure 4. Winter wheat weight of 1000 grains (g) in the following years of the experiment, depending on the biochar type. Different lowercase letters indicate statistically significant differences between biochar types.

3.2.2. Wet Gluten Content and Gluten Index

Gluten is a complex mixture of proteins found in wheat and related grains like barley and rye. It plays a crucial role in the baking industry due to its unique properties that contribute to the texture, structure, and overall quality of baked goods. Since nitrogen fertilization is one of the key aspects influencing gluten content [41], the amendment of biocarbons, which can hold and release nitrogen, might have a significant impact on gluten content. In the presented study, the total dose of biocarbon significantly differentiated the amount of wet gluten only in the first year of this study (Figure 5). The application of biocarbon at $3 \text{ t} \cdot \text{ha}^{-1}$ resulted in a higher value for this parameter. In the following years of this study, only a trend towards increased wet gluten amounts with increased biocarbon doses was observed. According to Khan et al. [34], the application of biocarbon at much higher rates than in the presented study ($20 \text{ t} \cdot \text{ha}^{-1}$) resulted in an increase in the protein content of wheat grain and straw. This was also confirmed by Selivanovskaya [42] for biochar used at $30 \text{ t} \cdot \text{ha}^{-1}$. Also, Shahzad et al. [43] found an increase (by $6.8 \text{ g} \cdot \text{kg}^{-1}$) in the protein content of wheat grain cultivated with biochar amendment. Kraska et al. [44] found that $20 \text{ t} \cdot \text{ha}^{-1}$ of biochar amendment resulted in a higher protein content of rye grain than biochar applied at $30 \text{ t} \cdot \text{ha}^{-1}$. In the presented study, the type of biocarbon significantly shaped the amount of wet gluten in winter wheat grain in 2018 and 2020 (Figure 6). In 2018, the best value for this indicator was found on the site where meat and bone meal biocarbon was used. In 2020, MPB biochar had the highest content of wet gluten. WCB biochar showed one of the highest wet gluten contents in both 2018 and 2020.

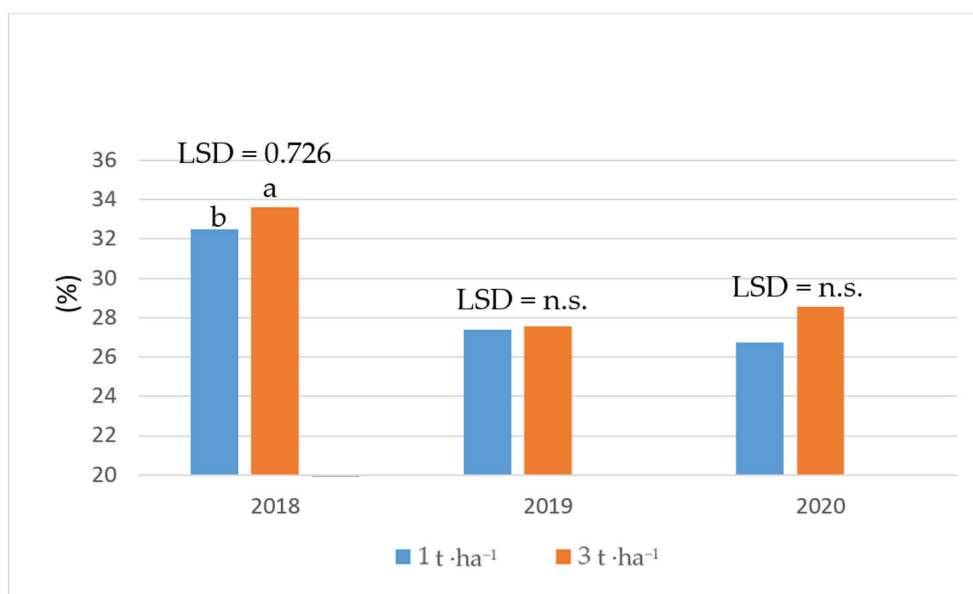


Figure 5. Winter wheat wet gluten content (%) in the following years of the experiment, depending on the biochar dose. Different lowercase letters indicate statistically significant differences between biochar doses.

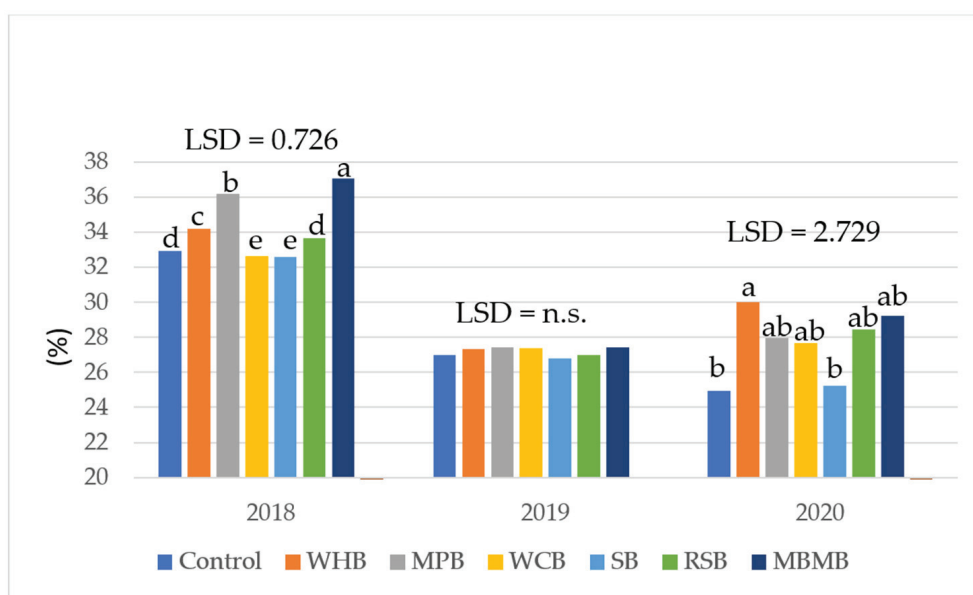


Figure 6. Winter wheat wet gluten content (%) in the following years of the experiment, depending on the biochar type. Different lowercase letters indicate statistically significant differences between biochar types.

The gluten index is a measure of the gluten strength in wheat flour. It provides information about the quality of gluten and its ability to form a stable and elastic network in dough. The gluten index is an important parameter in the baking industry, particularly in the production of bread and other baked goods. There was no significant effect of the biocarbon dose on the gluten index (Figure 7). There was only a trend toward better gluten quality with a higher biocarbon dose, but the differences were not statistically proven. In all years of this study, the type of biocarbon had an effect on the gluten index. However, the effect was not the same in all years. In 2018, the highest value of this parameter was found for the biocarbon amendment made from forestry waste (WCB). In 2019 and 2020, a

significantly higher value of this index was found in the objects where biocarbon derived from meat and bone meal was used (MBMB) (Figure 8).

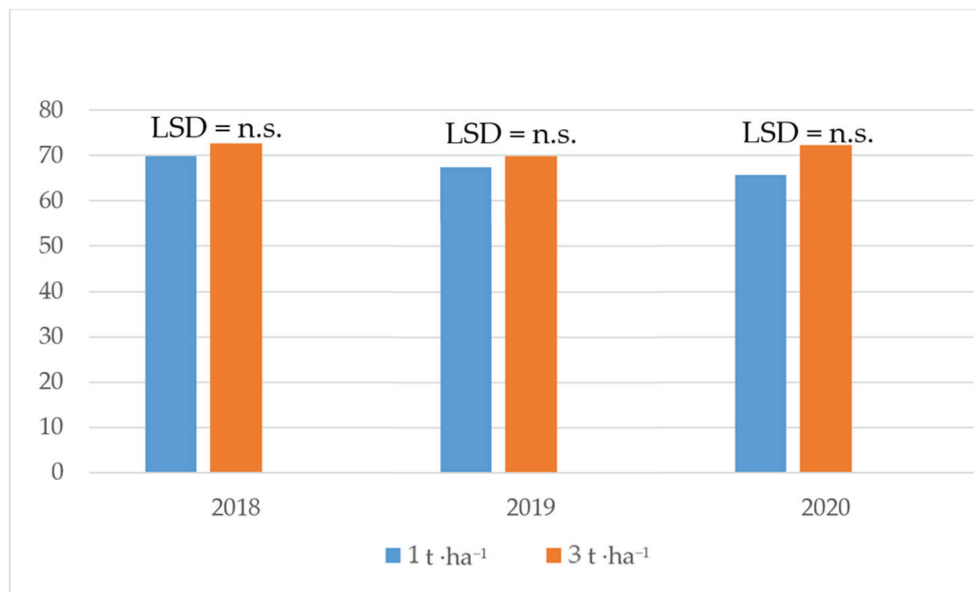


Figure 7. Winter wheat gluten index in the following years of the experiment, depending on the biochar dose.

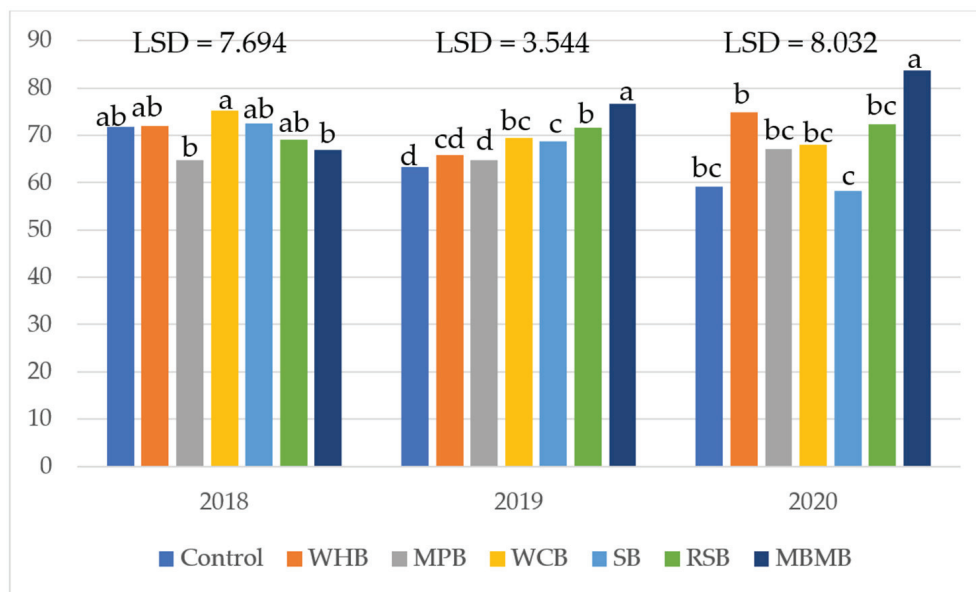


Figure 8. Winter wheat gluten index in the following years of the experiment, depending on the biochar type. Different lowercase letters indicate statistically significant differences between biochar types.

3.2.3. Grain Falling Number and Sedimentation Index

The falling number (FN) is a measure of alpha-amylase activity in grain and serves as an international standard of grain quality. It is determined through a test known as the Falling Number Test or Amylograph Test. This test is widely used in the flour milling and baking industries to assess the enzymatic activity that affects the quality of wheat and flour. High nitrogen fertilizer application can decrease the falling number due to increased alpha-amylase activity caused by the higher moisture of plant tissues. However, nitrogen application can also delay maturity, which may maintain a high falling number.

The relationship between nitrogen application and alpha-amylase activity is complex and context-dependent [45]. Excessive nitrogen, especially if applied late in the growing season, can increase the risk of pre-harvest sprouting and affect enzyme activity [46]. Moreover, grain falling can be affected by weather conditions. Barnard and Smith [47] found a negative impact of rainfall during the late stages of grain development on FN for some of the tested cultivars. The falling number, according to Hruskova et al. [48], can be strongly affected by weather conditions, especially higher temperatures, which can be positively correlated with FN. In the present study, FN showed a tendency to reach higher values in 2019, with temperatures in June being the highest of the three years. However, at the same time, the average temperature in April and May 2019 was lower than in 2018 and 2020 (Table 2). This might show that temperatures, especially at the last development stages, can be crucial for the development of quality parameters.

The Zelleny sedimentation index relates to both the quality and quantity of protein in the grain and therefore has an impact on the quality of the bread obtained and, in particular, its structure. Its higher values are desirable, which can be promoted by, for example, nitrogen fertilization [49]. A high sedimentation rate should be combined with a high content of gluten proteins, especially gluten itself, which is particularly important for the baking industry [50]. In the present study, there was no effect of biocarbon dose on the value of the falling number (Figure 9), however falling number was significantly influenced by biocarbon type in 2018 and 2019 (Figure 10). The value of the Zelleny sedimentation index was not influenced by biocarbon dose (Figure 11). However, the type of biocarbon used significantly differentiated the Zelleny sedimentation index values between tested objects (Figure 12). Differences in falling numbers between different biochar types were found for 2018 and 2019. In 2018, MBMB had the highest value of falling numbers, whereas in 2019, MBMB biochar had the lowest value of falling numbers. This indicates that this parameter was strongly affected by other factors than biochar amendment type, most likely weather conditions. The biochar type used had a significant impact on the Zelleny sedimentation index in all three years of this study. In 2018, all tested objects showed low values of the index; however, the highest values were observed for the control object and MBMB biochar. In 2019, SB biochar had the highest value of the Zelleny index (biochar with one of the highest concentrations of nitrogen). In 2019, the MBMB biochar showed the lowest value of this parameter. In 2020, RSB biochar had the highest Zelleny's sedimentation index (biochar with the lowest nitrogen content), while SB biochar had one of the highest values of this parameter. The results showed one more time that the impact of biochar type on grain quality parameters is visible but highly variable and dependent on external parameters. Zelleny's sedimentation index, as a grain-falling number, can be strongly influenced by weather conditions [51]. Stepień and Wojtkowiak [52] found that higher N fertilization rates positively impact Zelleny's sedimentation index. However, as in our study, biochars with the highest concentration of nitrogen (SB and RSB) had different results in Zelleny's sedimentation index in different years, indicating weather as the most important driving factor for Zelleny's sedimentation index values. As wheater conditions (minmal, maximal temperatures, rainfall, and humidity) have a strong impact on both falling number and sedimentation index, we assume that those parameters of wheat's grain were strongly influenced by both weather conditions in the following years of this study, as well as nitrogen availability (modified by the type of biocarbon used), hence the large variations in this factor between years and between the types of biocarbon used.

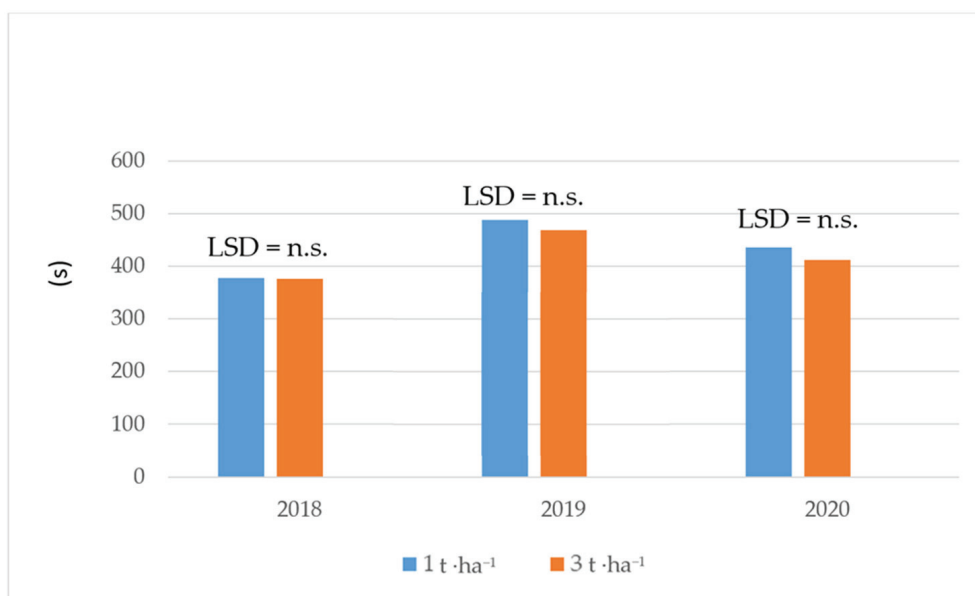


Figure 9. Winter wheat falling number (s) in the following years of the experiment, depending on the biochar dose.

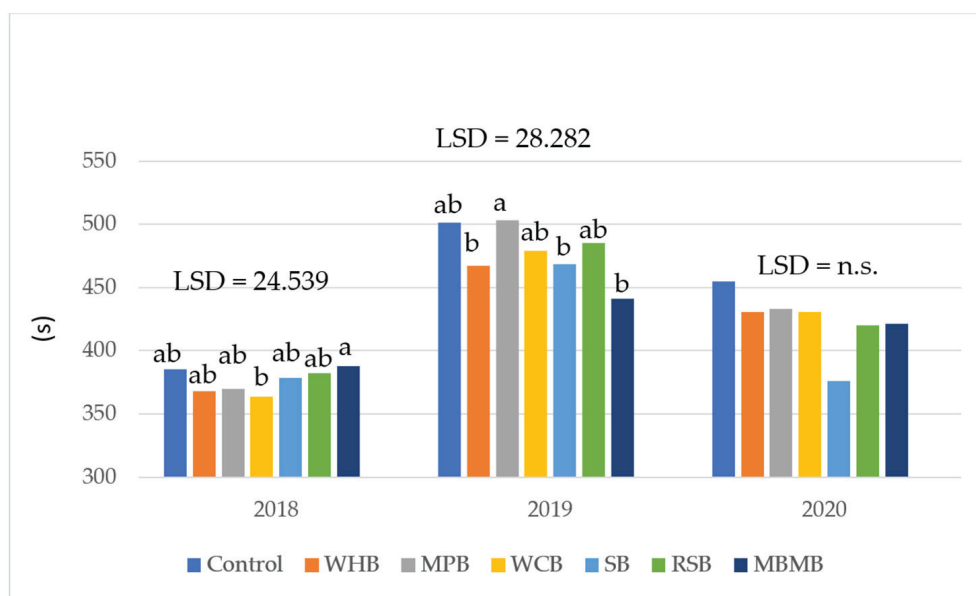


Figure 10. Winter wheat falling number (s) in the following years of the experiment, depending on the biochar type. Different lowercase letters indicate statistically significant differences between biochar types.

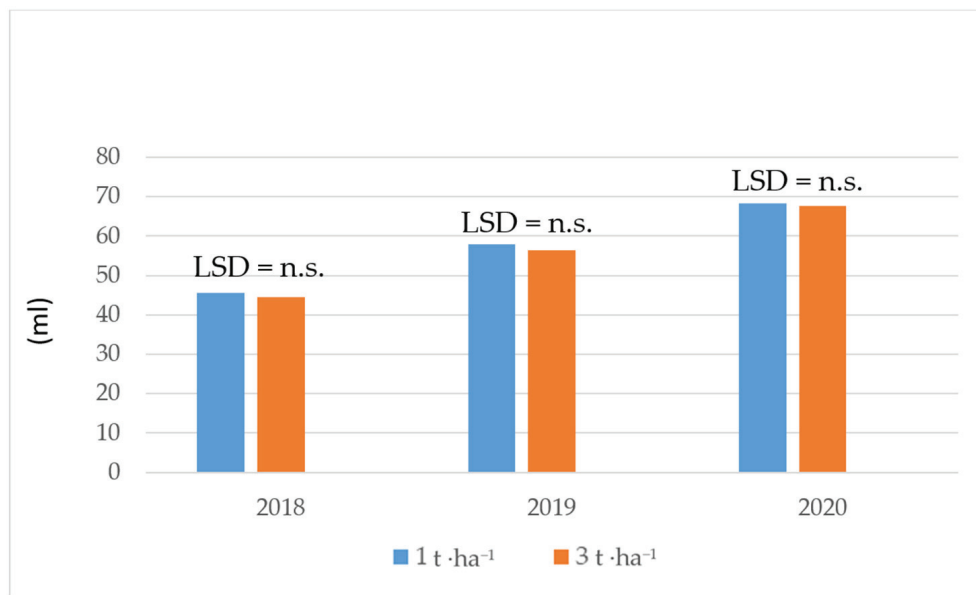


Figure 11. Winter wheat Zelleny sedimentation value (ml) in the following years of the experiment, depending on the biochar doses.

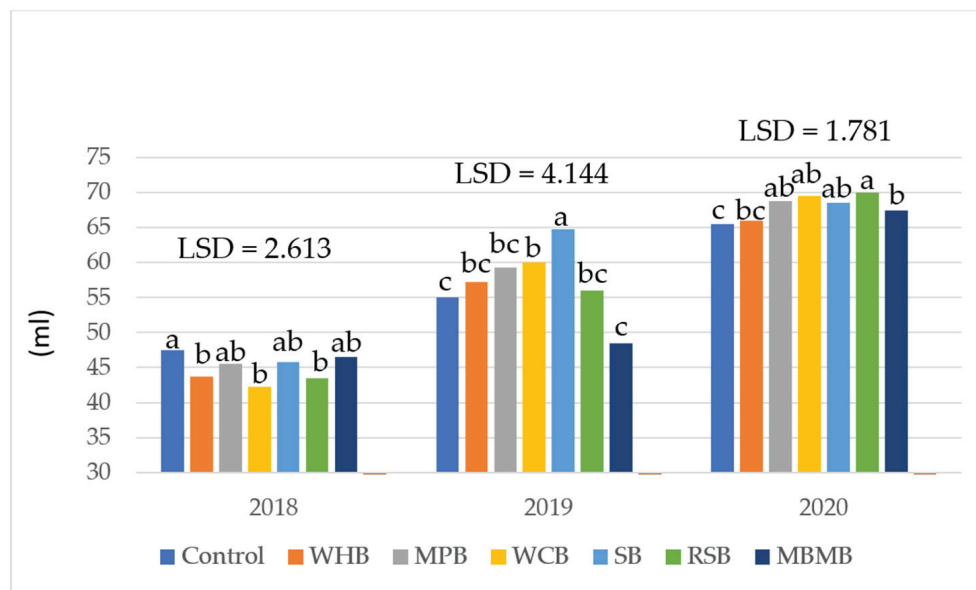


Figure 12. Winter wheat gluten value (ml) in the following years of the experiment, depending on the biochar type. Different lowercase letters indicate statistically significant differences between biochar types.

4. Conclusions

The dose of biocarbon had little effect on the quantity or quality of winter wheat grain. Of the parameters tested, only the gluten content showed a positive response to a higher dose of biocarbon. The lack of a significant response from the other factors tested may be indirectly due to the deliberate selection of low doses of biocarbon treatments. In the literature, tested biochar doses are often 20 tons or even 30 tons per hectare. These are doses at which the effect of biocarbon is pronounced, but the unit cost of applying this treatment makes this treatment not feasible for everyone (the cost of 1 tonne of biochar was estimated by Nematian et al. [53] at USD \$450, even up to USD \$1850). Lower doses seem to be more realistic and thus worth testing. The type of biocarbon used, which was inversely related to the dose, had a significant effect on many of the parameters tested. This

could have been influenced by the characteristics of the raw material, its physical properties (particularly the size of biochar particles) or chemical properties (carbon content, macro- and micronutrient content), and pH. SB biocarbon (Saw Dust biochar) showed rather good results for most of the parameters tested. Also, MBMB biochar showed interesting results, especially in terms of gluten content. It also showed a tendency to promote high grain yields. Those two types of biocarbons had different properties, especially in terms of carbon content. In addition, the MBMB was the biocarbon that were physically the finest (had the smallest size of particles), according to the authors' data (no confirmation of this is in the table). In the present study, those two types of biochar can be recommended for further testing. Especially the low carbon content and high Nitrogen and Phosphorus content are the two parameters that could make SB biochar valuable for agricultural production. Tomczyk et al. [20] found that C content is higher in biochars produced at higher pyrolysis temperatures. Lower C content promotes higher ash and volatile solid contents. Moreover, sawdust is a waste that could possibly be obtained easily, in high amounts, and with a high rate of uniformity.

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Foliar Fertilization of Crop Plants in Polish Agriculture

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Abstract: Foliar fertilization makes it possible to quickly supply plants with deficient nutrients, in case of both their deficiency in the soil and hindered uptake. Crops are characterized by varying nutrient requirements for micronutrients, as well as varying sensitivity to their deficiency. The paper presents practical aspects of the foliar feeding of plants with micronutrients using foliar fertilizers, and their general classification and characteristics. The requirements of basic crops (cereals, rapeseed and corn) for the application of micronutrient fertilization and the degree of their sensitivity to micronutrient deficiency were characterized. The market of foliar fertilizers and the directions of its development were evaluated. The possibilities of foliar fertilizers containing amino acids and silicon, and the possibility of using them for biofortification are presented. It was found that foliar fertilization is one of the most popular and effective methods for the biofortification of plants, as it allows the delivery of the right amount of specific elements in a specific stage of plant development and is thus an economical and environmentally safe procedure. In conclusion, the analysis of the foliar fertilizer market shows that its development is very dynamic, and foliar fertilization is becoming one of the basic elements in effective crop production. Further expansion of the range of foliar fertilizers produced should be expected in accordance with the growing expectations of agricultural producers.

Keywords: foliar fertilization; micronutrients; biofortification; cereals; corn; rapeseed

1. Introduction

The systematic growth of the world's population requires providing it with sufficient food [1]. With the shrinking in areas devoted to crop cultivation, the only possible method for meeting the nutritional needs of such a large population is to increase the yield per unit area [2]. This increase is associated with the depletion of nutrients contained in soils, by way of uptake by plants and dispersal in the environment, resulting in severe nutrient deficiencies. The way to make up for these deficiencies is to use fertilizers, especially mineral fertilizers, in which the components are most often found in readily available forms. The use of mineral fertilizers in a soil-based manner is linked to their transformation in the soil, which affects their availability, resulting in the excessive or insufficient uptake of nutrients by plants. These phenomena can affect the quantity and quality of the yield obtained and the loss of nutrients due to their leaching into the soil and their excessive volatilization into the atmosphere, causing increasing environmental pollution [3].

The aforementioned aspects and problems associated with the use of mineral fertilizers inspire the search for global solutions to increase the efficiency of the use of nutrients from fertilizers, enabling the production of sufficient quantities of good-quality food while maintaining a healthy environment. These goals are served by, among others, the UN Sustainable Development Strategy, which plans to eliminate hunger and all forms of malnutrition worldwide by 2030 [4], as well as the European Green Deal strategy [5], which additionally indicates that by 2030, nutrient losses should be reduced by at least 50% and fertilizer use should be reduced by a minimum of 20%.

Achieving these goals is possible, but it means developing more efficient and effective fertilizer systems. Their goal should be to increase agricultural productivity with less fertilizer inputs [6]. Of the fertilization methods currently in use, such expectations are largely met by the foliar delivery of nutrients, the effectiveness of which can be up to several times greater than that of soil application [7].

The delivery of nutrients with foliar fertilizers generally involves (1) the application of an aqueous solution to the plant surface in the form of droplets, (2) their retention on the leaf surface, (3) the absorption of the nutrient into living plant cells and (4) the translocation of the nutrient to areas where it can be used by the plant in its life processes. The efficiency of foliar fertilization largely depends on the absorption mechanisms of foliar-applied molecules, as they are affected by many environmental factors. There are review papers in the literature that focus on the mechanisms of penetration of foliar-applied nutrient solutions through the leaf surface [8–10].

Foliar fertilization is an increasingly common way in plant nutrition to provide nutrients to plants, and nowadays, it is also aimed at the following goals [10]: (1) increasing the concentration of components in plant foods with biofortification (enrichment), using fertilizers containing readily available forms of deficient elements (selenium, iodine, zinc, iron), the deficiency of which occurs in the diet of animals and humans and affects about 25% of the population; (2) improving the utilization of the supplied elements by introducing substances that increase their uptake, water uptake and resistance to stress caused by abiotic factors. These include biostimulants that activate plant metabolic processes, reduce stress and alleviate nutrient deficiencies. These are substances that are said to completely change crop production in the near future.

The growing demand for quality food products, resulting from a growing world population, requires the use of optimal solutions to provide plants with optimal conditions for their growth and development [11]. While the weather conditions that affect the course of vegetation and crop yields are difficult to predict and control [12], in the process of feeding plants, we have the opportunity to take measures to increase the availability of nutrients for plants.

Proper foliar fertilization is one of the indispensable elements of agrotechnology to achieve high yields. This method of fertilization makes it possible to provide plants with all the necessary nutrients in every stage of development. It is also an effective way to stimulate and realize the potential of a given plant. It is an excellent way to support the root system of plants during periods of intensive growth. It makes it possible to provide plants, in a quick and effective way, with missing components during periodic shortages, resulting from the inability to absorb these components from the soil. Through foliar fertilization, we can also selectively supply micronutrients to sensitive plant species, so we are able to optimally meet the nutritional needs of plants. Foliar fertilizers provide high efficiency of fertilization and thus allow us to reduce the amount of components we introduce into the environment.

It is assumed that foliar fertilizers, depending on the crop, the level of agrotechnique, soil quality, weather conditions and, very importantly, the quality of the products, give the possibility of obtaining a yield increase of several to several tens of percentage points [13–17]. By supplying the plant with the missing essential nutrients, we can influence the yield and its quality parameters, which was stated as early as 1972, more than 50 years ago [18]. Thanks to foliar fertilization, we can also enrich plants in selected mineral components [19].

The market of foliar fertilizers in Poland is growing dynamically, gaining importance. It is estimated that more than 2000 different fertilizer products are currently available, and there is a 10-fold increase in their quantity compared with 2009 [7]. The wide range of foliar fertilizers on the market allows agricultural producers to choose products tailored to their current needs and field situation. However, the effective use of these fertilizers requires expert knowledge of the possibilities and advisability of their application. Agricultural producers expect products that are simple to use, characterized by full solubility, easy to dose and well miscible with chemicals, if such mixtures are approved for use [20].

Nowadays, in addition to the high nutrient content of foliar fertilizers, fertilizer manufacturers enrich their composition with various substances of a stimulating nature or supporting plant metabolism (plant extracts, algae, vitamins or amino acids) [21–23]. This makes it possible to increase the plant's resistance to stress factors, diseases and pests, or to support plant regeneration after a stress factor. Preininger et al. [24] emphasize the positive effect of using bacteria, fungi or viruses on foliar fertilization of plants.

Currently, the rules for the introduction of foliar fertilizers to the Polish market are regulated by Polish regulations [25] and EU regulations on, inter alia, making fertilizer products available on the EU market [26]. According to the regulations, in order for a fertilizer to be approved for marketing, it must be made from raw materials that meet the requirements of one of the component material categories (CMCs) in Annex 2 [26]. So, among other things, what is important is the ingredient itself, its origin or its purity, while it is not important, for example, the particle size or chemical form of the element in question. In practice, most manufacturers aim to introduce and make their solutions available in accordance with the EU regulations (mainly due to the freedom of circulation in member countries), and if this is not possible, national regulations are used.

2. Discussion

2.1. Nutritional Demands of Cereals, Rapeseed and Corn

The nutritional requirements of cultivated crop species vary widely, and these needs are further influenced by the direction of production, habitat conditions, and weather. Significant differences in nutritional requirements also exist for varieties within a single species [27]. By using proper fertilization, we can also influence quality parameters, such as protein quality and content, gluten or sedimentation rate [28,29]. Therefore, it is very important that the size of fertilizer doses is adapted to the nutritional requirements of the plant species grown and the selected variety and that the current abundance of soils is taken into account, so that fertilization, which is intended to improve quantitative and qualitative parameters, does not have the opposite effect, i.e., a decrease in yield [30].

The contents of the forms of available nutrients in soils vary widely and depend on the species of soil, among other factors. Heavier soils, which contain more clay fractions, are characterized by a higher content of micronutrients compared with lighter, sandy soils [31]. Nutrient compactness can also be affected by tillage intensity, crop rotation and applied fertilization. In planning nutrient rates, it is important to know the content of nutrients in the soil in soluble (available) forms for plants. An example is the determination of plant doses of phosphorus, as it may turn out that despite the high content of the total form of this nutrient in the soil, up to 80% of it may be in a form that is not available to plants [32].

On the basis of long-term studies in Poland carried out by chemical and agricultural stations in cooperation with the Institute of Crop and Soil Sciences (IUNG) [31], a large share of soils with low abundance of micronutrients was found, especially for boron and copper (Table 1).

Table 1. Percentage of soils low in available forms of micronutrients in Poland.

Microelement	1987–1993	1994–1999	2000–2012	2016–2017	
				Wheat	Rapeseed
Boron (B)	75	79	74	19	45
Copper (Cu)	37	34	34	30	14
Iron (Fe)	-	28	21	21	10
Manganese (Mn)	11	7	3	16	15
Molybdenum (Mo)	23	-	-	-	-
Zinc (Zn)	14	13	17	20	14

- data not available.

Soil pH, which strongly influences the effectiveness of agrotechnical treatments, also has an impact on limiting the availability of components [33]. In 2016, about 72% of soils in Poland were characterized by very acidic or acidic pH (41% very acidic soils, 31% acidic soils); 19%, slightly acidic pH; and 9%, neutral pH [34]. Low soil pH contributes to an increase in the toxicity of aluminum ions to the root system, a reduction in the development of beneficial microorganisms, or the hydration and leaching of nutrients deep into the soil profile.

Fertilization, both in soil and foliar forms, should ensure that plants have access to nutrients in an amount that covers their nutritional requirements, especially in critical stages of their development. Stanislawska-Glubiak and Korzeniowska [31] indicate that crop plants differ significantly in their sensitivity to micronutrient deficiency (Table 2).

Table 2. Sensitivity of crop plants to micronutrient deficiencies.

Crop Plant	B	Cu	Mn	Mo	Zn
Wheat	1	3	3	0	1
Barley	0	3	2	1	0
Rye	0	0	2	0	0
Triticale	0	1	1	0	0
Oats	0	3	3	1	0
Rape	3	1	2	2	0
Sugar beet	3	2	3	2	1
Corn	2	2	2	0	3

0—none; 1—small; 2—medium; 3—large.

Among the basic nutrients, six macronutrients and six micronutrients can be specifically distinguished in terms of their indispensability to crop plants. From among the basic macronutrients such as nitrogen, phosphorus, potassium, magnesium, sulphur and calcium, plants take up the most nitrogen and potassium and this can be as much as over 200 kg per hectare. On the other hand, among the micronutrients boron, copper, iron, manganese, molybdenum and zinc, there is a wide variation in their requirements for plants. These needs range from a few to several hundred grams per hectare. It clearly follows that the main source of macronutrients for plants must be soil fertilization, while in the case of micronutrients, only foliar application can fully meet the nutritional needs of the plant [7].

2.2. Cereals

Cereals are among the most popular crops in Poland and also dominate global production. In Poland, in 2021, the area sown with cereals was 7.45 million hectares [35], and the popularity of this group of crops is due to the possibility of their versatile use. Cereal grains are used in the food and feed industries but can also be used in the energy or pharmaceutical industries [35]. The area of cereal crops in Poland has fluctuated over the past 50 years. The 1965–1980 period saw a decline in the area under cultivation, followed by an increase in interest in this group of crops between 1980 and 2000, and another decline since 2001. The 2000 season saw the largest area of cereal planting in Poland—8.81 million hectares [36].

Cereal cultivation is dominated by winter varieties, which have higher yields and better economic efficiency of production (Table 3). The estimated average yield of winter cereals in Poland in the 2021 season was 46 dt ha^{−1}, while that of spring cereals was 35 dt ha^{−1} [34].

Table 3. Cereal yield in Poland in 2010–2021.

Species of Cereal	2010	2015	2016	2017	2018	2019	2020	2021
	dt ha ^{−1}							
Basic Cereals with Cereal Mixtures	35.1	36.7	37.5	40.0	32.3	35.2	44.8	42.6
Winter wheat	45.7	47.6	47.2	51.1	43.0	46.4	54.2	51.8
Spring wheat	34.3	33.5	38.3	38.5	31.5	32.6	41.7	39.6
Rye	26.9	27.8	28.9	30.6	24.2	27.2	35.1	33.1
Winter barley	40.7	41.3	44.6	47.1	37.8	43.0	51.1	47.7
Spring barley	33.0	33.0	35.8	38.0	29.5	32.1	40.0	37.8
Oats	26.4	26.5	28.4	29.8	23.5	24.9	33.2	31.4
Winter triticale	35.2	36.3	37.1	40.4	32.8	35.9	45.0	43.1
Spring triticale	28.4	28.4	31.7	32.9	25.1	27.5	36.4	33.7
Winter cereal mixes	30.9	30.9	32.4	34.4	28.2	30.6	38.1	36.6
Spring cereal mixes	30.5	27.2	29.8	32.2	25.0	26.2	34.5	33.7

Different cereal species are characterized by different nutrient requirements. Winter wheat requires 22–26 kg of nitrogen (N), 8 kg of phosphorus (P₂O₅), 5 kg of potassium (K₂O), 2 kg of magnesium (MgO) and 1 kg of calcium (CaO) to produce 1 t of grain. In turn, this species' micronutrient requirements in grams per hectare are as follows: boron (B), 115 g; copper (Cu), 120 g; manganese (Mn), 500 g; molybdenum (Mo), 7 g; and zinc (Zn), 350 g [28].

2.3. Rapeseed

In recent years, there has been a steady increase in interest in the cultivation of rapeseed. In the 2021 season, the area under cultivation of this crop in Poland amounted to 0.99 million hectares [35]. In comparison, in 1947, the cultivation area was only 61 thousand hectares, and in 2002, 439 thousand hectares. With the increase in the area of cultivation of this plant, an increase in the yield of production per 1 ha is also noted. Over the past 10 years, the average yield per 1 ha has increased from 23.6 dt to 32.1 dt ha^{−1}, or as much as 36%. The increase in the yield of this crop is due to the introduction of new, improved varieties and improved agrotechnology, including fertilization. The high popularity of rapeseed, as in the case of cereals, is the result of significant market demand for this type of product. Among other uses, rapeseed is used in the production of cooking oils and in the production of feedstuffs, as well as biofuels [37].

Rapeseed is characterized by very high nutrient requirements. To produce 30 dt of rapeseed, it is necessary to provide 213 kg of nitrogen (N), 89 kg of phosphorus (P₂O₅), 287 kg of potassium (K₂O), 157 kg of calcium (CaO), 70 kg of nitrogen (MgO) and 75 kg of sulfur (S) [38]. Of the micronutrients, rapeseed shows the greatest sensitivity to and high demand for boron and manganese. The demand according to various authors ranges from 80 g ha^{−1} of boron and 100 g ha^{−1} of manganese [39] to 300 g of boron and 500 g of manganese for the assumed yield of 5 t ha^{−1}. Regarding other micronutrients, rapeseed needs about 50–200 g of copper, 300–750 g of zinc and 5–10 g of molybdenum for the assumed yield of 5 t ha^{−1} [40].

2.4. Corn

Corn is a crop of major economic importance worldwide [41]. The volume of yield and the acreage devoted to its cultivation place it among the three most important crops, next to wheat and rice [42]. It is also of great importance in our country, due to its multiple uses, mainly for grain and silage, but it can also be used in the production of biogas or biofuels. The last few decades have seen a significant increase in the area under cultivation. In Poland, in 1995, the species occupied an area of 181 tys. ha, while in 2009, the cultivation area increased to 695 tys. ha [43]. In 2019, the area of corn cultivated for grain was 665 tys,

and that for silage, 600 tys. ha, giving us a total of 1.26 million ha and placing it second, in the area of sown crops in Poland, after cereals and just ahead of rapeseed [35].

Table 4 summarizes the average yield of corn in Poland in the period from 2010 to 2020, with average grain yield ranging from 47.1 to 71.5 dt ha⁻¹, and green forage, from 357 to 493 dt ha⁻¹ (Table 4) [35].

Table 4. Corn yield in Poland.

Use of the Corn Crop	2010	2015	2016	2017	2018	2019	2020
	dt ha ⁻¹						
Grain	59.7	47.1	72.9	71.5	59.9	56.2	56.2
Forage	437	357	493	487	426	406	459

Corn is characterized by a sizable demand for nitrogen; it is assumed to require 25 kg of this element to produce one ton of grain and a corresponding weight of straw [44]. It is also characterized by a high demand for phosphorus and potassium. To produce 1 ton of grain with adequate straw, it requires about 10 kg of phosphorus (P₂O₅) and 30 kg of potassium (K₂O). Thus, for the yield of 10.0 tons of grain ha⁻¹, the nutritional needs are 250 kg of nitrogen (N), 100 kg of phosphorus (P₂O₅) and 300 kg of potassium (K₂O). Good sulfur supply for corn plays a key role in nitrogen utilization and conversion [45], enabling higher yield with less nitrogen fertilization. The beneficial effect of sulfur on increased plant uptake and utilization of the applied nutrient has also been reported [46]. Balanced plant nutrition should take into account the supply of both macronutrients and micronutrients to plants, which affect the efficiency of the uptake of the supplied components [47] but also contribute to the increase in the obtained yield themselves [48].

Corn is very sensitive to zinc and boron deficiencies, while it is less sensitive to deficiencies of copper, manganese and molybdenum. Fertilization with micronutrients is particularly important because corn is mainly grown on light soils, which are characterized by a much lower content of micronutrients compared with heavy soils. The requirement for corn to produce one ton of grain in relation to micronutrients is as follows: 20 g of boron (B), 12 g of copper (Cu), 45 g of iron (Fe), 35 g of manganese (Mn), 1 g of molybdenum (Mo) and 50 g of zinc (Zn). The use of micronutrients in corn production contributes to higher yield, but the effectiveness of micronutrient fertilization treatment depends, among other things, on the availability of macronutrients [48], indicating that the combined application of macro- and micronutrients produces better yield-forming effects [49].

2.5. Types of Foliar Fertilizers

The number of foliar fertilizers available on the Polish market has been steadily increasing for several years, mainly due to the growing demand of agricultural producers for dedicated, specialized products adapted to the requirements of individual crops. Scientific research confirming the applicability of the products in agricultural practice contributes to the increase. We can divide the available products into several different groups, depending on the selected criterion:

1. Physical form: (a) liquid fertilizers—in this group, we can distinguish among liquid fertilizers, fertilizers in suspension and gel; (b) loose fertilizers—soluble in water, they are in the form of powders and crystals of various shapes.
2. Purpose: (a) universal—fertilizers that can be used in any crop; (b) dedicated—tailored to the nutritional needs of selected plants and crops.
3. Amount of components in the fertilizer: (a) monocomponent—dominant, high content of one macro- or micronutrient (N, B, Cu, Zn, Mn); (b) bicomponent—high content of two components, whether macronutrients (N + Ca, P + K, S + Mg, N + K, etc.), micronutrients (B + Mo, B + Zn, B + Mn, Mn + Zn) or a mix of macro- and micronutrients (N + Mn, N + Mo, P + B, P + Zn); (c) multicomponent—containing a minimum of three or more nutrients (N + P + K, N + P + K + micro, P + K + Mg, Zn + B + Mg).

4. Type of components: (a) primary macronutrients with high content—N, P, K; (b) secondary macronutrients with high content—Ca, Mg, Na, S; (c) micronutrients with high content—B, Cu, Fe, Zn, Mn, Mo; (d) mixed fertilizers—fertilizer mixtures with increased content of selected macro- and micronutrients.

5. Forms of nutrients: (a) “pure” ionic forms; (b) complexed—with complexing agents, such as lignosulfonic acid (LS), glutamine hydroxamate (HGA), organic acids; (c) chelated—chelating agents, such as ethylenediaminetetraacetic acid (EDTA), 2-hydroxyethylethylenediaminetriacetic acid (HEEDTA), diethylenetriaminepentaacetic acid (DTPA), ethylenediamine-N,N′-bis(2-hydroxyphenylacetic) acid (EDDHA), ethylenediamine-di(o-hydroxy-o-methylphenylacetic) acid (EDDHMA), ethylenediamine di-(2-carboxy-5-hydroxyphenylacetic) acid (EDDCHA), ethylenediamine-N-N=bis(2-hydroxy-5-sulfophenylacetic) acid (EDDHSA), N-(1,2-dicarboxyethyl)-D,L-aspartic acid (IDHA), N,N-di(2-hydroxybenzyl)ethylenediamine-N,N-diacetic acid (HBED) and ethylenediaminedisuccinic acid (EDDS).

6. Homogeneity: (a) complex fertilizers—fertilizers containing at least two nutrients, characterized by the fact that in the solid phase, each granule has exactly the same content of each of the declared components; (b) blended fertilizers (blends)—fertilizers resulting from the physical mixing of at least two other fertilizers, without chemical reactions.

7. The content of additional components: (a) deficient components—silicon (Si), iodine (I), chromium (Cr); (b) adjuvants—supportive agents, improving the effectiveness of foliar fertilization treatments; (c) anti-stress substances—substances that increase resistance to stress factors (amino acids, plant extracts, vitamins); (d) stimulants—substances and chemical compounds that stimulate the plant for intensive development (e.g., amino acids, hormones, humic substances).

2.6. Foliar Fertilizer Market

The foliar fertilizer market in Poland is currently estimated at around PLN 300 million. The main suppliers of the products are Polish companies, but many solutions from Europe and further corners of the world are also available. We can count, among the leaders of the domestic market, the companies ADOB[®], EKOPOLON[®] and INTERMAG[®]. These are companies that have been engaged in the production of foliar fertilizers for more than 30 years, supplying their products both to the Polish market and to many other countries around the world. Among foreign manufacturers, the Polish market is dominated by European companies coming from Italy, France, Spain, Great Britain, Belgium and Turkey, but there are also companies from the United States of America, China or Japan. Every year, new domestic and foreign manufacturers of foliar fertilizers and biostimulants appear on the Polish market. Such a large number of suppliers allows Polish agricultural producers to benefit from the latest solutions in the field of foliar fertilization and biostimulation.

The market of foliar fertilizers has undergone quite a transformation over the past 30 years. In the 1990s, liquid fertilizers containing basic macro- and micronutrients dominated the market. Some of these products are still available on the market and do not promise to disappear in the near future. An example of such a product is liquid boron fertilizers containing boron in organic form—boroethanolamine—which account for about 70–80% of all boron fertilizers used on farms. The remaining 20–30% is in bulk, soluble forms based on boric acid and sodium borates. Blends of boron fertilizers with macro- and micronutrients are also available.

Between 2000 and 2010, loose soluble fertilizers began to appear and gain importance on the market. These were products mainly containing nitrogen, phosphorus and potassium, along with micronutrients. Four products were standard in the offer of manufacturers: balanced fertilizer, containing nitrogen, phosphorus, potassium and micronutrients at the same level; fertilizer with increased nitrogen content; fertilizer with increased phosphorus content; and fertilizer with increased potassium content. Compared with liquid fertilizers, bulk fertilizers require slightly longer preparation time due to the need for pre-dissolution. However, in many cases, they have a much higher content and concentration of nutri-

ents. In addition, they are cheaper to transport and more resistant to changing weather conditions during storage.

The biggest boom in the market of foliar fertilizers occurred after 2010, at which time, macro- and micronutrient products, enriched with various additives, began to appear. Currently, on the market, we have many innovative solutions tailored to the current specifics of production, plant requirements and changing environmental conditions.

In the tables below (Tables 5–7) is a comparison of selected fertilizers dedicated to the cultivation of cereals, rapeseed and corn, as well as popular directions in which producers and science are directing their research and development efforts.

Table 5. Selected foliar fertilizers used in cereal crops.

Foliar Fertilizer	Chemical Composition											Type of Complexes/Chelates
	N	P ₂ O ₅	K ₂ O	MgO	SO ₃	B	Cu	Fe	Mn	Mo	Zn	
Liquid fertilizers												
Ekolist cereals	127	38.1	38.1	-	-	0.13	11.4	8.9	10.1	0.06	8.9	EDTA organic complexes
Plonvit cereals	195	-	-	26	59	0.18	11.7	10.4	14.3	0.06	13	EDTA organic complexes
Vital cereals	198	-	-	66	-	1.32	3.3	6.6	11.8	0.16	13.2	No
Sarplon cereals	245	-	34.3	10	-	1.32	6.6	13.2	19.8	0.26	4	EDTA/DTPA
Suplofol Micro Z	204	-	-	27	68	2	6.8	13.6	25.9	0.2	13.6	No
Crystalline fertilizers												
Maximus amino micro cereals	11	-	70	-	-	3.4	50	20	40	0.4	20	Glicyna
Adob Micro cereals	100	-	50	-	310	-	15	3	30	0.2	5	EDTA
Amino Ultra cereals	-	-	-	20	-	1.6	16	65	65	0.7	49	Glicyna
Cereals forte	50	150	150	78	20	0.2	10	1	10	0.01	0.04	EDTA/DTPA
Dr Green cereals	-	-	-	-	-	5	50	60	80	0.5	20	Micro Activ
Opti cereals	140	160	160	30	180	-	3	1.5	5	0.4	1.5	EDTA/DTPA
Suspension fertilizers												
Yaravita Gramitrel	64	-	-	250	-	-	50	-	150	-	80	Oxide form

Table 6. Selected foliar fertilizers used in rapeseed cultivation.

Foliar Fertilizer	Chemical Composition											Type of Complexes/Chelates
	N	P ₂ O ₅	K ₂ O	MgO	SO ₃	B	Cu	Fe	Mn	Mo	Zn	
Liquid fertilizers												
Ekolist rape	150	50	37.5	-	-	8.7	0.1	8.7	8.7	0.06	0.12	Technology ACTIVE
Plonvit rape	186	-	-	31	31	6.2	1.2	6.2	6.2	0.06	6.2	Technology INT
Vital rape	188	-	-	40	-	6.9	1.2	3.1	4.4	0.09	3.7	No
Sarplon rape	285	-	30.4	12.4	1.45	5.3	2.38	2.38	16.8	0.53	2.4	EDTA/DTPA
Suplofol micro BR	195	-	-	26	65	6.2	0.85	1	23.4	0.2	9.5	No

Table 6. Cont.

Foliar Fertilizer	Chemical Composition											Type of Complexes/Chelates
	N	P ₂ O ₅	K ₂ O	MgO	SO ₃	B	Cu	Fe	Mn	Mo	Zn	
Crystalline fertilizers												
Maximus Amino Micro rape	110	-	70	-	-	20	15	30	40	0.4	15	Glicyna
Adob Micro rape	47	-	-	-	135	100	5	3	15	1	3	EDTA
Rapeforte	50	150	150	46	147	30	0.03	1.5	10	0.01	0.04	EDTA/DTPA
Dr Green rape	-	-	-	-	-	100	2	25	50	0.5	20	Micro Activ
OPTI rape	110	150	210	20	190	15	1	1.5	2	0.4	1.5	EDTA
Suspension fertilizers												
Yaravita Gramitrel	64	-	-	250	-	-	50	-	150	-	80	Oxide form

Table 7. Selected foliar fertilizers used in corn cultivation.

Foliar Fertilizer	Chemical Composition											Type of Complexes/Chelates
	N	P ₂ O ₅	K ₂ O	MgO	SO ₃	B	Cu	Fe	Mn	Mo	Zn	
Liquid fertilizers												
Ekolist corn	75.6	126	37.8	-	-	6.3	1.2	7.5	2.5	0.06	11.3	Technologia ACTIVE
Plonvit corn	195	-	-	26	54.6	5.2	7.8	9.1	9.1	0.065	14.3	Technologia INT
Vital corn	203	-	-	69	-	1.6	4.1	6.7	13.5	0.13	17.6	Brak
Sarplon corn	271	-	11.9	18.5	-	4	1.3	1.3	6.6	0.46	19.8	EDTA
Suplofol Micro K	188	-	-	25	63	2.5	1.25	3.75	5	0.38	20	No
Crystalline fertilizers												
Maximus Amino Micro corn	-	110	70	-	-	20	20	20	30	0.4	50	Glicyna
Adob Mikro corn	70	20	-	30	100	20	1	2	5	0.1	40	EDTA
Corn forte	50	200	150	42	220	15	0.07	1	0.1	0.01	15	EDTA. DTPA
Dr Green corn	-	-	-	-	-	5	2	60	70	0.5	80	Micro Activ
OPTI corn	100	210	140	30	140	5	2	1	0.3	3	1	EDTA
Suspension fertilizers												
YaraVita Zeatrel	-	440	75	67	-	-	-	-	-	-	46	Oxide form

From the compilation of fertilizers in the tables (Tables 5–7), each product, even if dedicated to the same crop, is characterized by a different composition. On the market, we have products that contain all basic macro- and micronutrients, as well as those that only contain selected nutrients. Individual products also differ in the content of additives, the main purpose of which is to improve the efficiency of fertilization and/or support the plant in case of biotic and abiotic stresses. Large discrepancies can also be seen in the recommended dosages and prices of individual fertilizers. The above comparison shows that when choosing a particular solution, many factors should be taken into account, including composition, forms of components and their availability to the plant, as well as dosage, the price of the fertilizer or the type of chelating, complexing substances, which should be selected to maximize the effectiveness of foliar fertilization under specific conditions in the field.

2.7. Foliar Fertilizers Containing Amino Acids

The last 10 years have seen a significant increase in interest in amino acids used in plant fertilization. Currently, on the market, we have many products containing amino acids. Depending on the product, the content of these components can vary from less than 1% to more than 40% of the total weight of the fertilizer [23]. The various solutions also differ in the source of amino acids, e.g., plant or animal origin, and in the content of individual amino acids. All amino acids except glycine can differ in optical activity. They can exist in L- and D-forms, and very importantly, both forms can be taken up and utilized by plants [50]. There have been many publications on the effectiveness of amino acids, with authors confirming the effectiveness of fertilizers containing amino acids of both plant and animal origin [51]. The effectiveness of amino acids is due, among other things, to their nitrogen content, which is necessary for optimal plant growth and development, as well as their effect on the efficiency of uptake and utilization of other nutrients [23]. They can also influence the content of chlorophyll or carotenoids in plants, key substances involved in photosynthesis [52,53].

2.8. Foliar Fertilizers Containing Nano-Elements

Among the products used in foliar fertilization are those that contain nanocomponents. Their effectiveness can depend on the timing of application, concentration or particle size [54]. These fertilizers are gaining importance due to their ability to reduce the negative impact of fertilizers on the environment and their effectiveness. A positive effect of nano-iron on fruit yield and quality parameters was shown by [55], while in an experiment with rapeseed, a yield increase of 1298 kg ha⁻¹ was reported [56]. Vishekaii et al. [57] studied the effect of boron nano-chelates on fruit and olive oil yield. Among the available literature, one can also find information on the positive effects on growth and yield of nano-molybdenum [58], nano-zinc [54] or nano-silicon [59], nano-copper, and nano-silver [60]. Meena et al. [61] studied the effects of nano-phosphorus, potassium and zinc on wheat cultivation, showing positive effects of nanoparticles on growth and yield at levels ranging from a few to tens of percentage points, depending on the combination.

2.9. Foliar Fertilizers Containing Silicon

For several years, the foliar fertilizer market has seen an increase in interest in foliar fertilizers containing silicon. Among other things, the use of these products contributes to the strengthening of plant cell walls, thereby increasing resistance to damage [62]. Foliar application of silicon also has a positive effect on reducing water loss through the leaves [63]. The positive effect of silicon on the response of plants to high temperatures was demonstrated in their experiment by Basirat and Mousavi [64]. The experiment was conducted in a greenhouse under controlled, high-temperature conditions (36 °C). Foliar application of silicon resulted in a 36.1% increase in total cucumber yield and a 40.3% increase in marketable yield. The positive effect of silicon applied in the form of nanoparticles was also demonstrated by Shalaby et al. [59]. In their study, they showed that the effectiveness of silicon fertilizers depended on the number of fertilization treatments and habitat conditions, and that yield gains could range from 6.4% (one treatment) to 12.9% (three treatments) in years with optimal rainfall and from 12.2% to 17.6% in dry years [63].

2.10. Foliar Fertilizers for Biofortification

The word biofortification is becoming more and more popular every year. There is more and more talk about deficiencies of selected elements in the diet of humans and animals and thus the need to develop effective methods for providing these elements [65]. One of the most effective ways is plant biofortification, that is, enriching plants with specific elements and improving their availability. The process of biofortification can take place via fertilization (topdressing and foliar) or breeding varieties that are able to accumulate increased amounts of selected elements. Foliar fertilization is one of the most popular and effective methods of plant biofortification, as it allows the delivery of the right amount of

specific elements in a specific developmental stage of the plant and is thus an economical and environmentally safe procedure.

Most often, iodine and selenium are mentioned in biofortification, but the topic can also apply to other nutrients. In the case of iodine, the minimum human requirement is $1 \mu\text{g kg}^{-1}$ body weight day^{-1} , and the optimal dose is $95\text{--}150 \mu\text{g day}^{-1}$. The human body has a similar requirement for selenium, where the optimal level for one person is $50\text{--}200 \mu\text{g day}^{-1}$ [19]. Currently, there are no foliar fertilizers for biofortification on the domestic market, but scientific research has been conducted for more than 10 years to determine the optimal doses and chemical forms of iodine or selenium. The effects of foliar and soil application of iodine and selenium in their experiments were studied by [66–69]. Biofortification using agrotechnical methods is a direction that will become increasingly important in the coming years, as the problem of nutrient deficiency in the diet of humans and animals affects not only our country but also the entire globe.

3. Conclusions

The analysis of the foliar fertilizer market in Poland indicates that its development is very dynamic. High competition among manufacturers of foliar fertilizers contributes to the appearance of many new innovative products on the market. Currently, products enriched with natural or synthetic additives to improve the efficiency of fertilization are expected to become the standard.

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Article

Perennial Ryegrass (*Lolium perenne* L.) Response to Different Forms of Sulfur Fertilizers

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Abstract: The aim of the study was to compare sulfate fertilizers and mixtures of elemental sulfur (S^0) and sulfate in terms of yield and nitrogen (N) and sulfur (S) status in perennial ryegrass. Mixtures of sulfate and S^0 can reduce the consumption of sulfate alone. The plants were grown in soil cultures. The plants were supplemented with S^0 , K_2SO_4 , $MgSO_4$, and $(NH_4)SO_4$ or a mixture of these salts with S^0 . Two sulfur doses were applied and the ryegrass was harvested three times. Fresh and dry weights of each swath, the N and S content, and their uptake were determined. The total fresh yield of sulfur-fertilized plants was 25 to 94% higher compared to unfertilized plants. The increases in dry matter were even more significant. Fertilizers, being a mixture of S^0 and sulfate, showed the same efficiency as those containing sulfate alone. Sulfur fertilization resulted in a higher S content and its uptake, lowered N concentration in second and third swath, and a decrease in total N uptake. In conclusion, to achieve high crop yields, soil sulfur deficiency should be corrected and fertilizers that are the mixture of elemental sulfur and sulfate are a beneficial and effective approach.

Keywords: elemental sulfur; sulfate; N:S ratio; ryegrass

1. Introduction

Sulfur (S), a macronutrient found in plants in the smallest amounts relative to other essential macronutrients, is currently recognized as one of the most important yield-forming elements [1,2]. In recent years, a shortage of plant-available sulfur in soils almost all over the world has been [3–6]. This adverse phenomenon is due to a drastic reduction in air pollution by sulfur, and consequently, a reduction in its deposition in the soil [6,7]. High-yielding crops grown today take up large amounts of sulfur from the soil and exacerbate this problem. It is expected that this phenomenon will intensify in the coming years [6,8] and in consequence, many agricultural areas will require fertilization with sulfur to maintain high yields and quality.

Therefore, research concerning sulfur application in agricultural plant production that will indicate an environmentally favorable, cheap for farmers, and efficient for plants solution of this problem is an urgent need. For proper growth and development, plants require sulfur at a level of 0.1–1.0% on a dry weight basis, and the average concentration of S in plant tissues ranges from 0.2 to 0.5% [9]. The main inorganic form of S directly available to plants is sulfate (SO_4^{2-}), which in mineral fertilizers, occurs as calcium, magnesium, potassium, or ammonium sulfate. Unfortunately, sulfate can be easily leached from the root zone, particularly from sandy soil. Furthermore, under sulfur limitation, plants absorb SO_4^{2-} very rapidly, which results in the formation of a sulfate depletion zone. On the other hand, sulfur is classified as an immobile mineral element in plants, which is not readily remobilized to younger leaves during deficiency. Its deficiency at any stage of plant

growth negatively affects plant metabolism and growth, and ultimately results in reduced yields [10]. This is due to the fact that sulfur is a multifunctional element which is not merely a component of certain amino acids (cysteine, methionine) and consequently proteins, but also builds other important cellular compounds [9,11]. They include coenzymes, lipids, and secondary metabolites that are involved in cellular metabolism, plant reaction to environmental stresses, and interactions with animals and pathogens [12]. Protein synthesis requires an adequate amount of two key macro-elements, nitrogen and sulfur; hence, a correct balance of these nutrients is particularly important [13].

Literature data indicate that sulfur and nitrogen metabolism are linked, and sulfur availability improves nitrogen uptake by plants, thereby affecting their quality and optimizing the N:S ratio [14,15]. The N:S ratio is considered to be a responsive indicator of sulfur supply to plants [16–19]. Fertilization with sulfur increases the total content of sulfur and sulfates in plants and deficit of this element results in the accumulation of non-protein nitrogen compounds that reduce the biological value of crop plants [15,20].

Fertilizers that are a mixture of sulfate and elemental sulfur (S^0) can be a good way to optimize sulfur supply to plants and improve its bioavailability in the long term. At the same time, they are cheaper and more environmentally friendly than sulfates alone. Elemental sulfur is chemically inert, difficult to leach from the soil compared to the anionic sulfate form, and available for longer in the soil. S^0 is a suitable source of this element for plants but must first be oxidized to the sulfate form by soil microorganisms. Oxidation of elemental sulfur and mineralization of organic sulfur, and thus the amount of available sulfur depends on the microbial activity of the soil. The temperature and moisture of the soil and its physico-chemical properties have a significant impact on this activity [11]. Sulfate is actively taken up through the roots and translocated to the shoot, and can be stored in vacuoles [9]. Plants are able to take up sulfate from the soil over a wide range of concentrations through the use of high-affinity and low-affinity transporters [11].

Literature data indicate that the application of sulfur increases the yield of crop and pasture plants and the magnitude of the response is dependent on the type of plant [21–23]. Some crops, such as oilseed rape and mustard, respond very well, while others respond much less well. There is little information on the response of meadow grasses; however, it is known that multi-cut grasses are more susceptible to sulfur deficiency than other crops [24]. In general, sulfur fertilization is expected to increase yields by an average of 25% under conditions of severe sulfur deficiency [25,26].

Perennial ryegrass—a species of the grass family—grows wild almost throughout Europe, northern Africa, the temperate zone of Asia, and was artificially introduced in North America and Australia. It is an excellent pasture grass that forms the basis of productive pastures for cattle. It can also be used to stabilize soil and prevent soil erosion, as well as to create hardy turf for lawns and golf courses. It has produced many varieties. Ryegrass is a highly productive plant and can be harvested multiple times during the growing season.

There is a relatively small number of studies that examine the sulfur fertilization needs of grasslands, especially in the temperate zone and over the past decade under conditions of very limited sulfur input from the atmosphere. Aspel et al. [27] in lysimeter experiments on perennial ryegrass showed that sulfur fertilization increased crop yields, nitrogen recovery from fertilizers, and significantly reduced nitrate leaching.

The aim of the study was to compare sulfate fertilizers and a mixture of elemental sulfur (S^0) and sulfate in terms of yield and N and S status in perennial ryegrass.

2. Materials and Methods

2.1. Pot Experiment

Plants were grown in pots filled with 2.5 kg of a sandy Arenosol whose granulometric composition was as follows: sand 86% with dominant medium and fine fractions, silt 12%, and clay 2%. The total content of carbon determined by the Dumas dry combustion method [28] and total content of sulfur determined by the Butters–Chenery method [29]

were very low (Table 1). The plant-available phosphorus, potassium, magnesium, and sulfate sulfur form content in the soil are given in Table 1. The soil had a very acidic pH prior to liming, a high content of phosphorus, and a low content of potassium and magnesium. Before sowing, the soil acidity was adjusted to pHKCl 6.62 by applying lime at a rate CaCO_3 (2.12 g kg^{-1}). The level of total sulfur content determined in the soil, along with S-SO_4 , classifies this soil type as low sulfur soil. Macronutrients were applied to the soil before sowing in the following doses: N 104 mg kg^{-1} , P 124 mg kg^{-1} , K 293 mg kg^{-1} , and Mg 92 mg kg^{-1} . The amounts of applied doses of macronutrients (N, P, K, Mg) were balanced taking into account the amount added with examined sulfates so that they were even and the same in all pots.

Table 1. Physical-chemical soil properties before the experiment.

Agronomic Category/Soil Texture	pH	C _{total}	N _{total}	S _{total}	P	K	Mg	S-SO ₄
					Soluble Forms			
	1M KCl dm^{-3}	g kg^{-1} soil		mg kg^{-1}		mg kg^{-1} Soil		
Light/Loamy sand	3.9	6.18	0.57	115	74	78	13	6.82

2.2. Schedule of Experience

Two sulfur doses of 60 and 120 mg S kg^{-1} soil were applied in the form of elemental sulfur (S^0), sulfates (K_2SO_4 , MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$), and a mixture of elemental sulfur and sulfates. The following treatments were established: no S—control soil without sulfur fertilization; S^0 —elemental sulfur application in the form of Wigor S fertilizer (“Siarkopol” Tarnobrzeg Co. Ltd., Poland); $\text{S}^0 + \text{K}_2\text{SO}_4$; K_2SO_4 alone; $\text{S}^0 + \text{MgSO}_4$; MgSO_4 ; $\text{S}^0 + (\text{NH}_4)_2\text{SO}_4$; $(\text{NH}_4)_2\text{SO}_4$. Each treatment included four repetitions (pots).

2.3. Cultivated Plant and Its Vegetation

In the pot experiment, perennial ryegrass (*Lolium perenne* L. variety Solen) was cultivated. About 100 seeds (0.3 g) of perennial ryegrass were sown in 20 holes and covered with $\sim 0.5 \text{ cm}$ of soil. Soil moisture was maintained at 60% water-holding capacity by adding deionized water. Plants were grown in a growth chamber under controlled conditions (photoperiod 16 h/8 h light/dark and temperature $26\text{--}28^\circ\text{C}/16\text{--}18^\circ\text{C}$ day/night). The grass was cut after 30, 45, and 60 days of vegetation.

2.4. Sample Preparation and Methods for Chemical Analysis

Representative soil samples were taken before and after vegetation experiments. Soil pH was determined by the potentiometric method using 1 mol dm^{-3} KCl, the total C and N content by analyzer (LECO) [28], total S content by the Butters–Chenery method [29], sulfate(VI) content by the Bardsley and Lancaster method [30], soluble forms of phosphorus and potassium by the Egner–Riehm method [31] and magnesium by the method in [32].

Plants were collected after each grass cutting and the fresh mass of ryegrass was determined. Then, plants were dried at 105°C for one hour (to kill plant tissues and avoid dry matter loss through respiration) and then at 60°C to constant weight. Total nitrogen level was determined by the combustion method [28], and total S content by the Butters–Chenery method [29].

2.5. Statistical Analysis

All results obtained were subjected to one-way analysis of variance. Prior to performing the analysis of variance, a test for homogeneity of variance within groups was performed using Levene’s test and the Shapiro–Wilk test of variables’ conformity to normal distribution. The significance of differences between the averages was assessed using the Tukey’s post hoc test with a significance level of $p < 0.05$. For all statistical analyses, the statistical program R was used [33].

3. Results and Discussion

3.1. Fresh and Dry Mass

Under the influence of 60 mg sulfur, the highest fresh and dry total mass was found for ammonium sulfate, and the observed increases were 94 and 139%, respectively, compared to the control (without S fertilization) (Figure 1). Similar increases were observed for the mixtures of elemental sulfur and sulfate ($S^0 + K_2SO_4$, $S^0 + MgSO_4$). Remaining S fertilizers also resulted in a statistically significant stimulation of ryegrass growth but not so spectacular. Elemental sulfur alone increased the total fresh and dry mass of ryegrass by 25 and 33%, respectively.

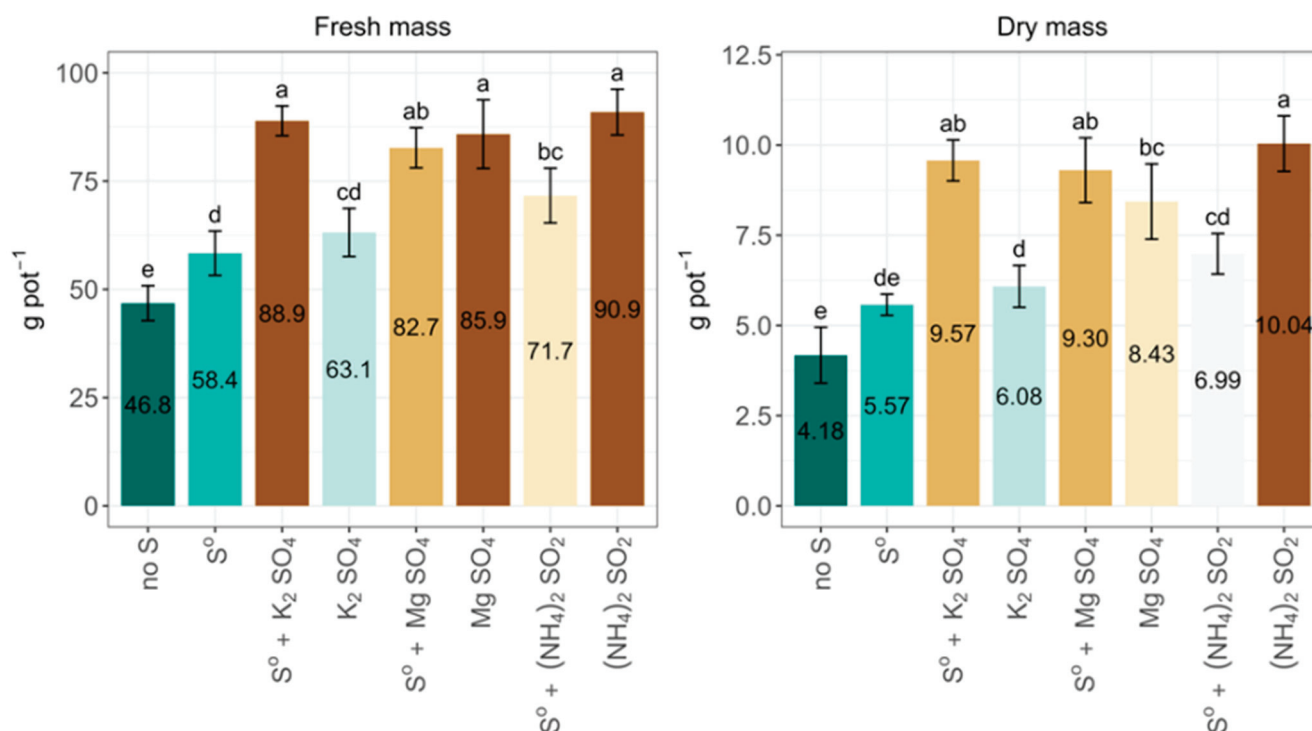


Figure 1. Total fresh and dry mass of ryegrass depending on sulfur fertilizer at a rate of S 60 mg/kg soil. Values labeled with the same letter are not significantly different ($p < 0.05$).

The response of ryegrass to the higher dose of sulfur (120 mg) was similar to the response to the lower dose and the least effective fertilizers were elemental sulfur and a combination of S^0 and $(NH_4)_2SO_4$ (Figure 2). These results show that ryegrass reacts very well to sulfur fertilization and a mixture of S^0 and sulfate gives significantly better results than the use of elemental sulfur alone and comparable results with the use of sulfates alone.

Figure 3 shows the dry matter yield of ryegrass obtained on the three harvest dates depending on the form of sulfur fertilizer used and the dosage rate of this element. In all the terms of ryegrass harvest, the dry matter yield obtained on fertilizers containing $S^0 + S-SO_4$ was similar to the objects where only sulfate forms were fertilized. The exception was the object with $S^0 + (NH_4)_2SO_4$ at the first harvest date, where significantly lower yields of ryegrass were obtained. As the vegetation period lengthened, the differences in ryegrass dry matter yields between the fertilizers applied decreased.

In order to visualize the differences between the fertilizers used, they were grouped according to the form of sulfur they contained (Figure 4). In all harvest dates, the combination of the $S^0 + S-SO_4$ form allowed dry matter yields to be obtained that did not differ from those obtained when fertilizing with the sulfate form alone.

Dry matter yields of ryegrass were also compared in relation to the sulfur doses applied (Figure 5). It was found that there were no significant differences between the

sulfur doses used, either in the total dry matter yield or in the yields obtained at different harvest periods.

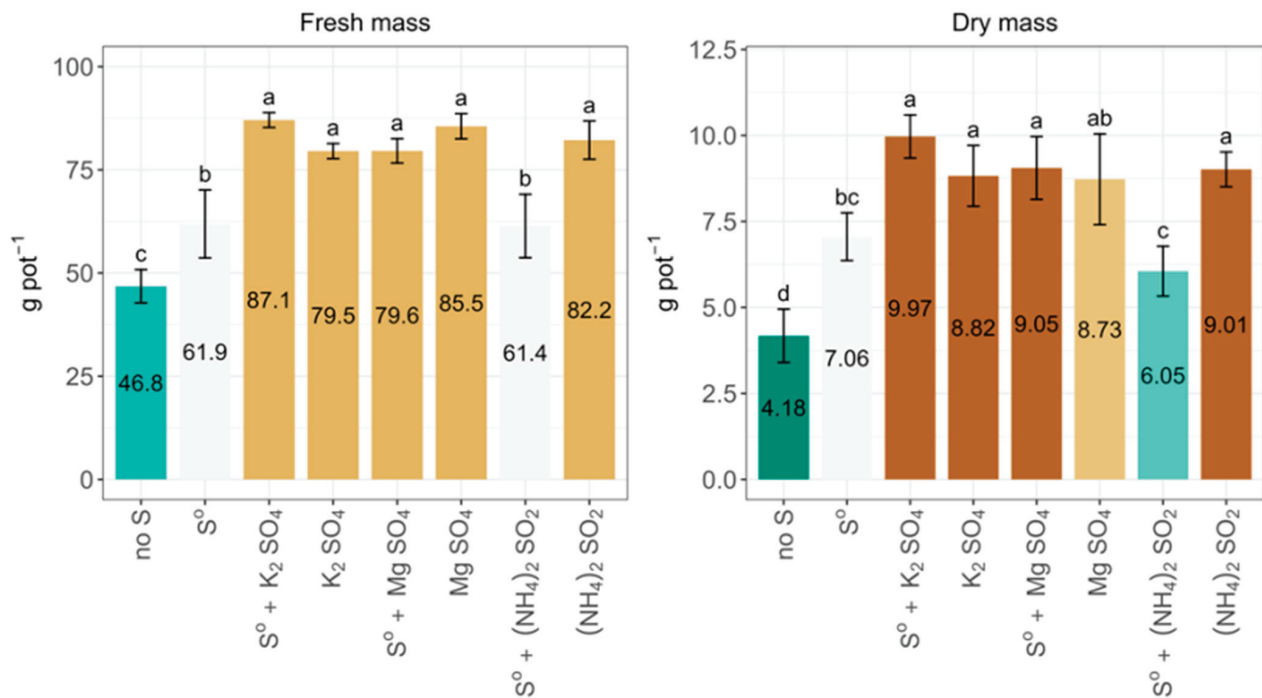


Figure 2. Total fresh and dry mass of ryegrass depending on S fertilizer at a rate of S 120 mg/kg soil. Values labeled with the same letter are not significantly different ($p < 0.05$).

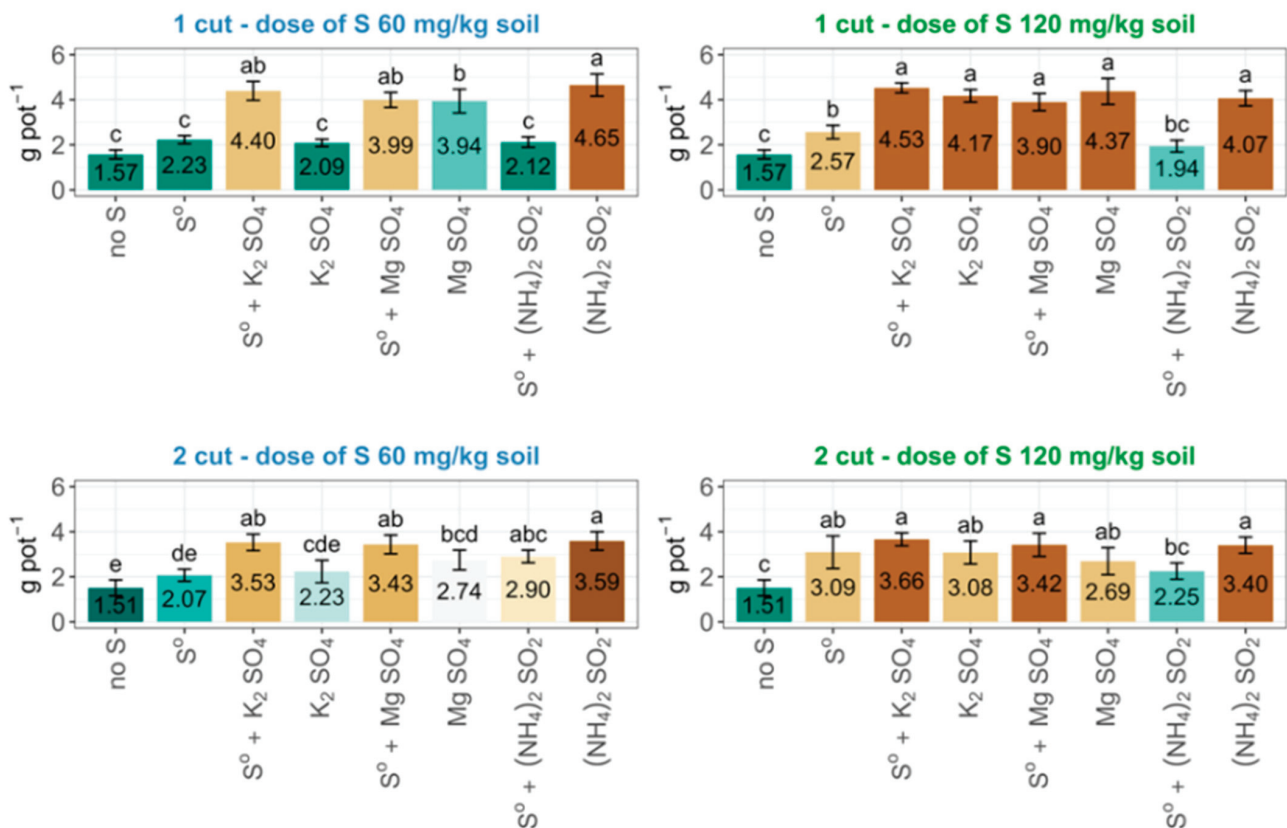


Figure 3. Cont.

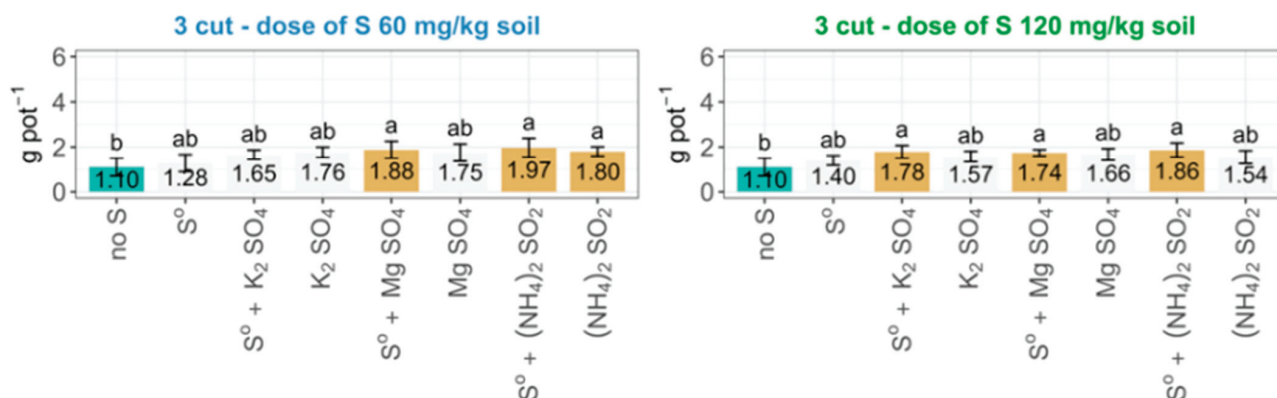


Figure 3. Dry mass of ryegrass depending on S fertilizer. Values labeled with the same letter are not significantly different ($p < 0.05$).

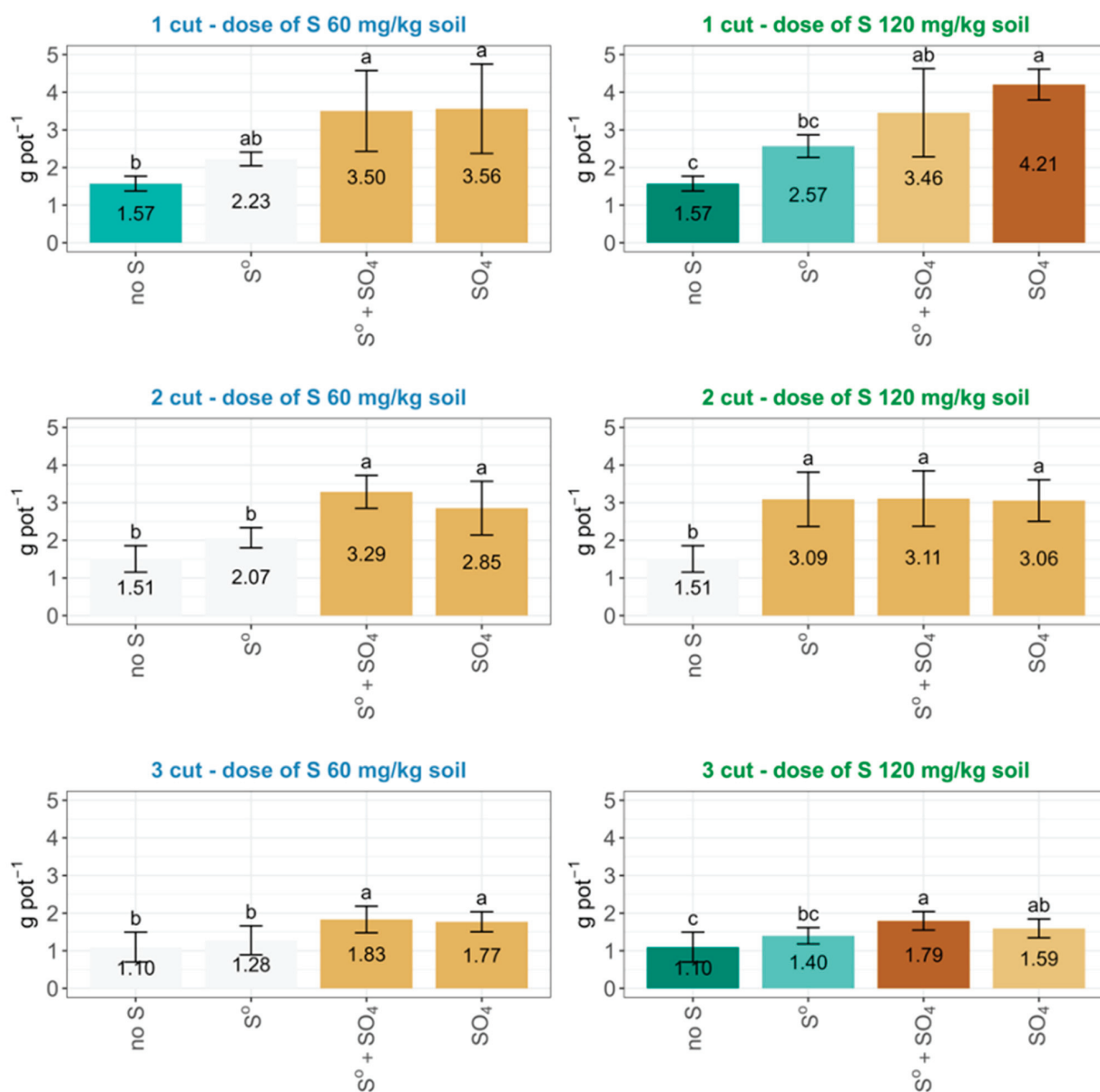


Figure 4. Comparison of dry mass of ryegrass according to forms of S. Values labeled with the same letter are not significantly different ($p < 0.05$).

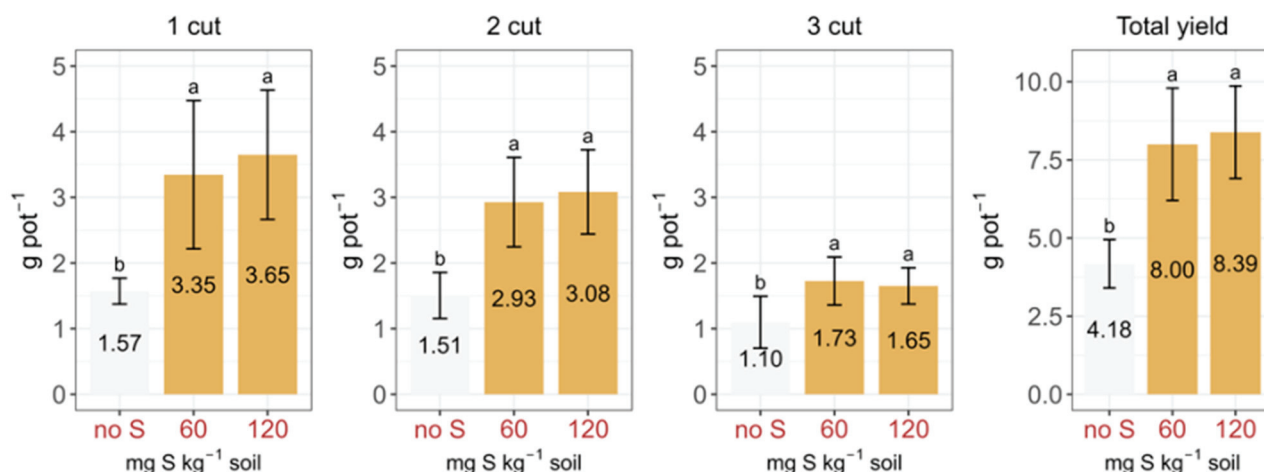


Figure 5. Dry mass of ryegrass in relation to S dose. Values labeled with the same letter are not significantly different ($p < 0.05$).

Degryse et al. [34] claim that both elemental sulfur and S^0 -enriched sulfate fertilizers sustain plant sulfur requirements over a longer period than SO_4 -S alone. Of the three swaths made in our experiment, the last one was significantly smaller than the earlier ones. This may have been due to the depletion of mineral nutrients in the soil as a result of their intensive uptake by the fast-growing grass. In the early stages of growth, the mineral requirements of plants are very high. Taube et al. [35] also found a varying response of successive regrowths of ryegrass to sulfur and nitrogen fertilization, with the greatest effect in the first regrowth. The results suggest that improved sulfur availability as a result of fertilization increases plant vigor, improves plant metabolism and photosynthetic activity, and ultimately results in better growth, higher stress tolerance, and improved nitrogen use efficiency. Our results are consistent with other studies that report a significant yield response of different crops to sulfur fertilization [25,27,34,36]. However, there are also reports indicating that plant growth response to sulfur addition is not always so positive and unequivocal [17,37]. Under field conditions, the response of plants to sulfur application is complex (among other immobilization, mineralization, leaching processes may have great impact) and, despite numerous studies, the exact requirements for sulfur fertilization are not known [34].

3.2. Nitrogen Content and Uptake

Nitrogen is one of the most important yield forming factors in agriculture. Literature data indicate that plant nitrogen content is significantly modified by the plant-available sulfur in the soil [27,37]. The results showed that the nitrogen content varied from one swath to the next, with the smallest differences (mostly statistically insignificant) occurring in the first swath (Table 2).

Under all applied treatments (form and dose of sulfur), the N concentration in the plants from the first swath ranged from 40.1 to 44.7 g kg⁻¹ dry matter. Plants from the first and second swaths contained significantly more nitrogen compared to the third swath. In the latter, under sulfur fertilization conditions, N concentration ranged from 19 g kg⁻¹ dry matter (for 120 mg S^0 + K_2SO_4) to 36.8 g kg⁻¹ dry matter (for 120 mg S^0) and the response to sulfur fertilization was similar for both sulfur doses. In plants not fertilized with sulfur, N concentrations were 42, 55.3, and 45.9 g kg⁻¹ dry mass in the first, second, and third swaths, respectively. Thus, nitrogen concentration in plants grown without sulfur addition was significantly higher than in plants fertilized with sulfur. The observed decrease was probably due to a dilution effect caused by the strong stimulation of ryegrass growth by sulfur fertilizers. As previously mentioned, the application of sulfur resulted in a significant increase in fresh and dry mass of ryegrass shoots (Figures 1 and 2). A decrease in the N content under sulfur fertilization was also observed in other plants [38]. Under

S⁰ treatment, the average N content of the three swaths was similar to that recorded in the unfertilized plants and not significantly different from the sulfate-fertilized plants.

Table 2. Nitrogen content in perennial ryegrass.

Treatments	N Content (g kg ⁻¹ Dry Mass)							
	1 Cut		2 Cut		3 Cut		Mean in Cuts	
Sulfur dose 60 mg kg ⁻¹ soil								
no S	42.0	<i>ab</i>	55.3	<i>a</i>	45.9	<i>a</i>	47.7	<i>a</i>
S ⁰	42.5	<i>ab</i>	54.8	<i>a</i>	34.5	<i>b</i>	43.9	<i>ab</i>
S ⁰ + K ₂ SO ₄	43.1	<i>ab</i>	39.1	<i>d</i>	19.8	<i>d</i>	34.0	<i>e</i>
K ₂ SO ₄	42.4	<i>ab</i>	50.1	<i>abc</i>	29.8	<i>b</i>	40.8	<i>bc</i>
S ⁰ + MgSO ₄	40.1	<i>b</i>	45.3	<i>bcd</i>	20.2	<i>cd</i>	35.2	<i>de</i>
MgSO ₄	43.9	<i>ab</i>	53.3	<i>a</i>	29.0	<i>bc</i>	42.1	<i>bc</i>
S ⁰ + (NH ₄) ₂ SO ₄	45.9	<i>a</i>	52.0	<i>ab</i>	29.5	<i>b</i>	42.5	<i>bc</i>
(NH ₄) ₂ SO ₄	42.8	<i>ab</i>	43.5	<i>cd</i>	30.4	<i>b</i>	38.9	<i>cd</i>
Comparison of sulfur forms for dose 60 mg kg ⁻¹ soil								
no S	42.0	<i>a</i>	55.3	<i>a</i>	45.9	<i>a</i>	47.7	<i>a</i>
S ⁰	42.5	<i>a</i>	54.8	<i>a</i>	34.5	<i>b</i>	43.9	<i>ab</i>
S ⁰ + SO ₄	43.0	<i>a</i>	45.5	<i>b</i>	23.2	<i>c</i>	37.2	<i>c</i>
SO ₄	43.0	<i>a</i>	49.0	<i>ab</i>	29.7	<i>b</i>	40.6	<i>b</i>
Sulfur dose 120 mg kg ⁻¹ soil								
no S	42.0	<i>a</i>	55.3	<i>a</i>	45.9	<i>a</i>	47.7	<i>a</i>
S ⁰	41.5	<i>a</i>	50.6	<i>a</i>	36.8	<i>b</i>	43.0	<i>ab</i>
S ⁰ + K ₂ SO ₄	40.9	<i>a</i>	33.3	<i>c</i>	19.0	<i>d</i>	31.1	<i>c</i>
K ₂ SO ₄	42.5	<i>a</i>	50.6	<i>a</i>	28.2	<i>c</i>	40.4	<i>b</i>
S ⁰ + MgSO ₄	40.6	<i>a</i>	37.5	<i>bc</i>	21.7	<i>cd</i>	33.3	<i>c</i>
MgSO ₄	43.3	<i>a</i>	46.8	<i>ab</i>	28.2	<i>c</i>	39.4	<i>b</i>
S ⁰ + (NH ₄) ₂ SO ₄	44.7	<i>a</i>	50.1	<i>a</i>	36.4	<i>b</i>	43.7	<i>ab</i>
(NH ₄) ₂ SO ₄	44.5	<i>a</i>	48.1	<i>a</i>	29.5	<i>bc</i>	40.7	<i>b</i>
Comparison of sulfur forms for dose 120 mg kg ⁻¹ soil								
no S	42.0	<i>a</i>	55.3	<i>a</i>	45.9	<i>a</i>	47.7	<i>a</i>
S ⁰	41.5	<i>a</i>	50.6	<i>a</i>	36.8	<i>ab</i>	43.0	<i>ab</i>
S ^o +SO ₄	42.1	<i>a</i>	40.3	<i>b</i>	25.7	<i>c</i>	36.0	<i>c</i>
SO ₄	43.4	<i>a</i>	48.5	<i>a</i>	28.6	<i>bc</i>	40.2	<i>b</i>
Comparison of sulfur doses: 60 and 120 mg kg ⁻¹ soil								
no S	42.0	<i>a</i>	55.3	<i>a</i>	45.9	<i>a</i>	47.7	<i>a</i>
60 mg kg ⁻¹ soil	43.0	<i>a</i>	48.3	<i>ab</i>	27.6	<i>b</i>	39.6	<i>b</i>
120 mg kg ⁻¹ soil	42.6	<i>a</i>	45.3	<i>b</i>	28.5	<i>b</i>	38.8	<i>b</i>

Values labeled with the same letter are not significantly different ($p < 0.05$).

The uptake of nitrogen by plants from individual swaths showed a similar pattern to that observed in nitrogen concentration (Table 3). The lowest nitrogen uptake was recorded in the third swath, and this amount was several times lower than in the first swath.

This downward effect was probably due to the depletion of readily available nitrogen in the soil. The highest total uptake (sum of three swaths) was recorded with (NH₄)₂SO₄ application (both S doses) and the lowest with no sulfur fertilization. Sulfur fertilization markedly increased nitrogen uptake by ryegrass plants, which in turn contributed to a significant stimulation of plant growth. In general, the effect of both sulfur doses was similar, with the exception of the S⁰ fertilization where the total nitrogen uptake was significantly greater under the higher S dose, and counted 303 mg pot⁻¹ against 245 for the lower dose. Reports on the interaction between N and S suggest that they affect each other, from the soil uptake, transport in the plant, and assimilation in cells [14]. Sulfur-deficient crops utilize nitrogen inefficiently, which results in increased losses of nitrogen to the environment [39,40].

Table 3. Nitrogen uptake of perennial ryegrass.

Treatments	N Uptake (mg pot ⁻¹)							
	1 Cut		2 Cut		3 Cut		Total Uptake	
Sulfur dose 60 mg kg ⁻¹ soil								
no S	66.0	<i>c</i>	82.8	<i>d</i>	50.4	<i>a</i>	199	<i>d</i>
S ⁰	94.6	<i>c</i>	113	<i>bcd</i>	44.2	<i>a</i>	245	<i>cd</i>
S ⁰ + K ₂ SO ₄	189	<i>ab</i>	138	<i>abc</i>	32.5	<i>a</i>	325	<i>b</i>
K ₂ SO ₄	88.6	<i>c</i>	110	<i>cd</i>	53.3	<i>a</i>	248	<i>cd</i>
S ⁰ + MgSO ₄	160	<i>b</i>	155	<i>a</i>	36.6	<i>a</i>	327	<i>ab</i>
MgSO ₄	174	<i>ab</i>	147	<i>abc</i>	51.1	<i>a</i>	356	<i>ab</i>
S ⁰ + (NH ₄) ₂ SO ₄	97.3	<i>c</i>	151	<i>ab</i>	56.5	<i>a</i>	296	<i>bc</i>
(NH ₄) ₂ SO ₄	199	<i>a</i>	156	<i>a</i>	54.3	<i>a</i>	389	<i>a</i>
Comparison of sulfur forms for dose 60 mg kg ⁻¹ soil								
no S	66.0	<i>c</i>	82.8	<i>c</i>	50.4	<i>a</i>	199	<i>b</i>
S ⁰	94.6	<i>bc</i>	113	<i>bc</i>	44.2	<i>a</i>	252	<i>b</i>
S ⁰ + SO ₄	149	<i>ab</i>	148	<i>a</i>	41.9	<i>a</i>	339	<i>a</i>
SO ₄	154	<i>a</i>	138	<i>ab</i>	52.9	<i>a</i>	344	<i>a</i>
Sulfur dose 120 mg kg ⁻¹ soil								
no S	66.0	<i>d</i>	82.8	<i>c</i>	50.4	<i>ab</i>	199	<i>d</i>
S ⁰	107	<i>c</i>	157	<i>ab</i>	51.3	<i>ab</i>	303	<i>bc</i>
S ⁰ + K ₂ SO ₄	185	<i>ab</i>	121	<i>abc</i>	33.6	<i>b</i>	308	<i>abc</i>
K ₂ SO ₄	178	<i>ab</i>	154	<i>ab</i>	44.1	<i>b</i>	355	<i>ab</i>
S ⁰ + MgSO ₄	158	<i>b</i>	125	<i>abc</i>	37.5	<i>b</i>	299	<i>bc</i>
MgSO ₄	189	<i>a</i>	126	<i>abc</i>	46.2	<i>ab</i>	342	<i>ab</i>
S ⁰ + (NH ₄) ₂ SO ₄	86.8	<i>cd</i>	113	<i>bc</i>	67.7	<i>a</i>	265	<i>c</i>
(NH ₄) ₂ SO ₄	181	<i>ab</i>	164	<i>a</i>	45.6	<i>ab</i>	367	<i>a</i>
Comparison of sulfur forms for dose 120 mg kg ⁻¹ soil								
no S	66.0	<i>c</i>	82.8	<i>c</i>	50.4	<i>a</i>	199	<i>c</i>
S ⁰	107	<i>bc</i>	157	<i>a</i>	51.3	<i>a</i>	315	<i>b</i>
S ⁰ + SO ₄	143	<i>b</i>	120	<i>b</i>	46.3	<i>a</i>	309	<i>b</i>
SO ₄	183	<i>a</i>	148	<i>a</i>	45.3	<i>a</i>	376	<i>a</i>
Comparison of sulfur doses: 60 and 120 mg kg ⁻¹ soil								
no S	66	<i>b</i>	83	<i>b</i>	50.4	<i>a</i>	199	<i>b</i>
60 mg kg ⁻¹ soil	143	<i>a</i>	139	<i>a</i>	46.9	<i>a</i>	329	<i>a</i>
120 mg kg ⁻¹ soil	155	<i>a</i>	137	<i>a</i>	46.6	<i>a</i>	339	<i>a</i>

Values labeled with the same letter are not significantly different ($p < 0.05$).

3.3. Sulfur Content and Uptake

Overall, as expected, sulfur fertilization resulted in a better supply of sulfur to the ryegrass, and in some cases, the effect was more pronounced with a higher dose of sulfur. This was particularly evident after the application of fertilizers containing only sulfates. Under these conditions, the average sulfur content of the three swaths was 5.66 and 7.0 g kg⁻¹ dry mass for the 60 and 120 mg S kg⁻¹ soil doses, respectively (Table 4).

The highest sulfur concentration (as an average of three swaths) was recorded for plants fertilized with potassium sulfate (120 mg kg⁻¹ soil) and this value (8.02 g kg⁻¹ dry mass) was 70% higher than that of unfertilized plants. Fertilizing the plants with the mixture of elemental sulfur with potassium or magnesium sulfate did not increase the sulfur content in ryegrass shoots. Some positive effects were found only in the first swath. However, these were sufficient amounts for proper growth and function. Our results also indicate that ryegrass is capable of maintaining relatively high sulfur concentrations in shoots (4.74 g kg⁻¹ dry mass) despite low soil content. The recorded concentration is within the range expected for most crops (0.2 to 0.5% dry matter) [9]. However, a negative consequence was a strong inhibition of plant growth compared to sulfur-fertilized plants. In terms of sulfur requirements, multiple-cut grasses and Brassica crops are more prone to sulfur deficiency than other plants [20]. The sulfur demand is dependent not only on the plant species but also on its developmental stage [41]. During vegetative growth, sulfur uptake is optimized for growth. All plants regulate sulfur uptake and are able to adjust to

very variable (low and high soil sulfur levels) and time-varying sulfur supply [42]. The uptake of sulfur by unfertilized plants remained at a similar level in the next three swaths (6.41, 7.27, and 5.71 mg pot⁻¹, respectively; Table 5). As expected, sulfur-fertilized plants took up significantly larger amounts of sulfur and, in general, the values were higher at the increased S dose. Analyzing the dynamics of sulfur uptake by S-fertilized plants, it can be stated that it was greatest during the first two periods of growth (Table 5).

Table 4. Sulfur content in perennial ryegrass.

Treatments	S Content (g kg ⁻¹ Dry Mass) in Perennial Ryegrass							
	1 Cut		2 Cut		3 Cut		Mean in Cuts	
Sulfur dose 60 mg kg ⁻¹ soil								
no S	4.09	<i>b</i>	4.84	<i>d</i>	5.28	<i>bc</i>	4.74	<i>d</i>
S ⁰	4.88	<i>a</i>	5.62	<i>bc</i>	6.27	<i>ab</i>	5.59	<i>abc</i>
S ⁰ + K ₂ SO ₄	4.72	<i>ab</i>	5.40	<i>bcd</i>	4.16	<i>cd</i>	4.76	<i>d</i>
K ₂ SO ₄	4.94	<i>a</i>	6.01	<i>ab</i>	7.33	<i>a</i>	6.09	<i>a</i>
S ⁰ + MgSO ₄	4.79	<i>a</i>	5.58	<i>bc</i>	3.96	<i>d</i>	4.78	<i>d</i>
MgSO ₄	4.55	<i>ab</i>	5.40	<i>bcd</i>	5.45	<i>b</i>	5.13	<i>cd</i>
S ⁰ + (NH ₄) ₂ SO ₄	4.93	<i>a</i>	5.25	<i>cd</i>	5.86	<i>b</i>	5.35	<i>bc</i>
(NH ₄) ₂ SO ₄	4.93	<i>a</i>	6.30	<i>a</i>	6.04	<i>b</i>	5.76	<i>ab</i>
Comparison of sulfur forms for dose 60 mg kg ⁻¹ soil								
no S	4.09	<i>b</i>	4.84	<i>c</i>	5.28	<i>ab</i>	4.74	<i>b</i>
S ⁰	4.88	<i>a</i>	5.62	<i>ab</i>	6.27	<i>a</i>	5.59	<i>a</i>
S ⁰ + SO ₄	4.81	<i>a</i>	5.41	<i>b</i>	4.66	<i>b</i>	4.96	<i>b</i>
SO ₄	4.80	<i>a</i>	5.90	<i>a</i>	6.27	<i>a</i>	5.66	<i>a</i>
Sulfur dose 120 mg kg ⁻¹ soil								
no S	4.09	<i>e</i>	4.84	<i>cd</i>	5.28	<i>de</i>	4.74	<i>d</i>
S ⁰	5.37	<i>c</i>	5.53	<i>bc</i>	6.77	<i>c</i>	5.89	<i>c</i>
S ⁰ + K ₂ SO ₄	4.96	<i>cd</i>	4.50	<i>d</i>	4.71	<i>e</i>	4.72	<i>d</i>
K ₂ SO ₄	6.88	<i>a</i>	6.00	<i>b</i>	11.2	<i>a</i>	8.02	<i>a</i>
S ⁰ + MgSO ₄	4.78	<i>d</i>	5.34	<i>bc</i>	4.43	<i>e</i>	4.85	<i>d</i>
MgSO ₄	5.15	<i>cd</i>	5.77	<i>b</i>	5.63	<i>cde</i>	5.52	<i>c</i>
S ⁰ + (NH ₄) ₂ SO ₄	5.48	<i>c</i>	5.30	<i>bcd</i>	6.06	<i>cd</i>	5.61	<i>c</i>
(NH ₄) ₂ SO ₄	6.21	<i>b</i>	7.58	<i>a</i>	8.56	<i>b</i>	7.45	<i>b</i>
Comparison of sulfur forms for dose 120 mg kg ⁻¹ soil								
no S	4.09	<i>c</i>	4.84	<i>b</i>	5.28	<i>b</i>	4.74	<i>b</i>
S ⁰	5.37	<i>ab</i>	5.53	<i>ab</i>	6.77	<i>ab</i>	5.89	<i>b</i>
S ⁰ + SO ₄	5.07	<i>b</i>	5.04	<i>b</i>	5.07	<i>b</i>	5.06	<i>b</i>
SO ₄	6.08	<i>a</i>	6.45	<i>a</i>	8.46	<i>a</i>	7.00	<i>a</i>
Comparison of Sulfur doses: 60 and 120 mg kg ⁻¹ soil								
no S	4.09	<i>c</i>	4.84	<i>b</i>	5.28	<i>b</i>	4.74	<i>b</i>
60 mg kg ⁻¹ soil	4.82	<i>b</i>	5.65	<i>ab</i>	5.58	<i>b</i>	5.35	<i>b</i>
120 mg kg ⁻¹ soil	5.55	<i>a</i>	5.72	<i>a</i>	6.76	<i>a</i>	6.01	<i>a</i>

Values labeled with the same letter are not significantly different ($p < 0.05$).

For sulfur fertilization at 60 mg S kg⁻¹ soil, the highest total uptake (56.4 mg pot⁻¹, almost 3 times higher compared to the unfertilized plants) was observed in plants fertilized with ammonium sulfate. In plants exposed to 120 mg S, the highest sulfur uptake was found in the presence of potassium sulfate and ammonium sulfate.

The assimilation of sulfur and nitrogen are strongly linked, so a proper balance of these nutrients is very important. The N/S ratio is used to diagnose sulfur deficiency in plants [43–45]. Empirically, it has been established that for every 15 parts of N in a protein, there is 1 part of S. This means that the N/S ratio is fixed within a very narrow range of 15:1. Sulfur deficiency can limit protein synthesis, even if a large amount of N is available. This relationship has very important implications for human and animal nutrition.

Our results showed that for all fertilization variants, the N/S ratio was below the critical value of 15:1 (Table 6). For the averages of the three swaths, the ratio ranged from 5.82 to 10.3 and this highest value relates to non-fertilized plants. It may be considered that

the recorded N/S ratios (all below 15:1) were satisfactory. Similar trends were observed for both sulfur doses. The reduced N/S ratio indicates that the plants did not suffer from sulfur deficiency (based on the value of the N/S ratio), and that plants not fertilized with sulfur were able to take up sulfur despite the low soil content. However, the growth of plants was significantly affected.

Table 5. Sulfur uptake of perennial ryegrass.

Treatments	S Uptake (mg pot ⁻¹)							
	1 Cut		2 Cut		3 Cut		Total Uptake	
Sulfur dose 60 mg kg ⁻¹ soil								
no S	6.41	<i>d</i>	7.27	<i>d</i>	5.71	<i>e</i>	19.4	<i>e</i>
S ⁰	10.9	<i>c</i>	11.6	<i>cd</i>	7.90	<i>cde</i>	30.4	<i>d</i>
S ⁰ + K ₂ SO ₄	20.7	<i>ab</i>	19.0	<i>ab</i>	6.85	<i>de</i>	46.5	<i>b</i>
K ₂ SO ₄	10.4	<i>cd</i>	13.5	<i>c</i>	12.9	<i>a</i>	36.7	<i>cd</i>
S ⁰ + MgSO ₄	19.2	<i>ab</i>	19.2	<i>ab</i>	7.21	<i>de</i>	45.5	<i>b</i>
MgSO ₄	17.9	<i>b</i>	14.8	<i>bc</i>	9.40	<i>bcd</i>	42.1	<i>bc</i>
S ⁰ + (NH ₄) ₂ SO ₄	10.4	<i>c</i>	15.2	<i>bc</i>	11.3	<i>ab</i>	36.9	<i>cd</i>
(NH ₄) ₂ SO ₄	22.9	<i>a</i>	22.6	<i>a</i>	10.9	<i>abc</i>	56.4	<i>a</i>
Comparison of sulfur forms for dose 60 mg kg ⁻¹ soil								
no S	6.41	<i>b</i>	7.27	<i>b</i>	5.71	<i>b</i>	19.4	<i>b</i>
S ⁰	10.9	<i>ab</i>	11.6	<i>b</i>	7.90	<i>b</i>	30.4	<i>b</i>
S ⁰ + SO ₄	16.8	<i>a</i>	17.8	<i>a</i>	8.46	<i>b</i>	43.0	<i>a</i>
SO ₄	17.1	<i>a</i>	17.0	<i>a</i>	11.0	<i>a</i>	45.1	<i>a</i>
Sulfur dose 120 mg kg ⁻¹ soil								
no S	6.41	<i>e</i>	7.27	<i>d</i>	5.71	<i>d</i>	19.4	<i>d</i>
S ⁰	13.8	<i>d</i>	17.0	<i>bc</i>	9.38	<i>bcd</i>	40.2	<i>bc</i>
S ⁰ + K ₂ SO ₄	22.4	<i>bc</i>	16.4	<i>bc</i>	8.49	<i>cd</i>	47.4	<i>b</i>
K ₂ SO ₄	28.7	<i>a</i>	18.5	<i>b</i>	17.5	<i>a</i>	64.8	<i>a</i>
S ⁰ + MgSO ₄	18.5	<i>c</i>	18.0	<i>b</i>	7.67	<i>cd</i>	44.2	<i>b</i>
MgSO ₄	22.5	<i>bc</i>	15.5	<i>bc</i>	9.21	<i>bcd</i>	47.3	<i>b</i>
S ⁰ + (NH ₄) ₂ SO ₄	10.6	<i>de</i>	11.9	<i>cd</i>	11.3	<i>bc</i>	33.8	<i>c</i>
(NH ₄) ₂ SO ₄	25.3	<i>ab</i>	25.7	<i>a</i>	13.4	<i>ab</i>	64.4	<i>a</i>
Comparison of sulfur forms for dose 120 mg kg ⁻¹ soil								
no S	6.41	<i>c</i>	7.27	<i>c</i>	5.71	<i>b</i>	19.4	<i>c</i>
S ⁰	13.8	<i>b</i>	17.0	<i>ab</i>	9.38	<i>ab</i>	40.2	<i>b</i>
S ⁰ + SO ₄	17.2	<i>b</i>	15.4	<i>b</i>	9.15	<i>b</i>	41.8	<i>b</i>
SO ₄	25.5	<i>a</i>	19.9	<i>a</i>	13.4	<i>a</i>	58.8	<i>a</i>
Comparison of sulfur doses: 60 and 120 mg kg ⁻¹ soil								
no S	6.41	<i>c</i>	7.27	<i>b</i>	5.71	<i>b</i>	19.4	<i>c</i>
60 mg kg ⁻¹ soil	16.1	<i>b</i>	16.5	<i>a</i>	9.49	<i>a</i>	42.1	<i>b</i>
120 mg kg ⁻¹ soil	20.3	<i>a</i>	17.6	<i>a</i>	11.0	<i>a</i>	48.9	<i>a</i>

Values labeled with the same letter are not significantly different ($p < 0.05$).

The lowest N/S ratio value was found in the last swath, probably due to a reduced pool of plant-available nitrogen in the soil and its limited uptake (Table 5). In the third swath, N/S values ranged between 2.52 (K₂SO₄ in a higher dose) and 8.74 (unfertilized plants). Analyzing the results, it can be concluded that an N/S ratio below 9.0 provides an optimum yield of perennial ryegrass. Ref. [27], in determining the potential of sulfur fertilization to increase grassland yields and N use efficiency, found that the N/S ratio was variable during the growing season. Its values ranged from 5:1 to 20:1 depending on the harvest date and the applied sulfur fertilization. Ref. [46] claims that S supplementation is more necessary at higher N doses.

Table 6. N:S ratio in perennial ryegrass.

Treatments	N:S in Perennial Ryegrass							
	1 Cut		2 Cut		3 Cut		Mean in Cuts	
Sulfur dose 60 mg kg ⁻¹ soil								
no S	10.3	<i>a</i>	11.4	<i>a</i>	8.74	<i>a</i>	10.3	<i>a</i>
S ⁰	8.76	<i>bc</i>	9.78	<i>b</i>	5.52	<i>b</i>	8.30	<i>bc</i>
S ⁰ + K ₂ SO ₄	9.15	<i>abc</i>	7.26	<i>c</i>	4.81	<i>b</i>	7.73	<i>cd</i>
K ₂ SO ₄	8.62	<i>bc</i>	8.39	<i>bc</i>	4.10	<i>b</i>	6.92	<i>d</i>
S ⁰ + MgSO ₄	8.42	<i>c</i>	8.11	<i>c</i>	5.10	<i>b</i>	7.75	<i>cd</i>
MgSO ₄	9.66	<i>ab</i>	9.91	<i>ab</i>	5.42	<i>b</i>	8.81	<i>b</i>
S ⁰ + (NH ₄) ₂ SO ₄	9.32	<i>abc</i>	9.92	<i>ab</i>	5.02	<i>b</i>	8.25	<i>bc</i>
(NH ₄) ₂ SO ₄	8.69	<i>bc</i>	6.91	<i>c</i>	5.01	<i>b</i>	7.25	<i>d</i>
Comparison of sulfur forms for dose 60 mg kg ⁻¹ soil								
no S	10.3	<i>a</i>	11.4	<i>a</i>	8.74	<i>a</i>	10.3	<i>a</i>
S ⁰	8.76	<i>b</i>	9.78	<i>ab</i>	5.52	<i>b</i>	8.30	<i>b</i>
S ⁰ + SO ₄	8.96	<i>b</i>	8.43	<i>b</i>	4.98	<i>b</i>	7.91	<i>b</i>
SO ₄	8.99	<i>b</i>	8.40	<i>b</i>	4.84	<i>b</i>	7.66	<i>b</i>
Sulfur dose 120 mg kg ⁻¹ soil								
no S	10.3	<i>a</i>	11.4	<i>a</i>	8.74	<i>a</i>	10.3	<i>a</i>
S ⁰	7.75	<i>bc</i>	9.18	<i>bc</i>	5.49	<i>bc</i>	7.81	<i>b</i>
S ⁰ + K ₂ SO ₄	8.26	<i>bc</i>	7.38	<i>cde</i>	4.10	<i>cd</i>	7.18	<i>b</i>
K ₂ SO ₄	6.17	<i>d</i>	8.52	<i>bcd</i>	2.52	<i>e</i>	5.82	<i>c</i>
S ⁰ + MgSO ₄	8.53	<i>b</i>	6.99	<i>de</i>	4.97	<i>bc</i>	7.27	<i>b</i>
MgSO ₄	8.41	<i>b</i>	8.15	<i>bcde</i>	5.02	<i>bc</i>	7.66	<i>b</i>
S ⁰ + (NH ₄) ₂ SO ₄	8.19	<i>bc</i>	9.47	<i>b</i>	6.00	<i>b</i>	7.91	<i>b</i>
(NH ₄) ₂ SO ₄	7.19	<i>cd</i>	6.36	<i>e</i>	3.47	<i>de</i>	6.05	<i>c</i>
Comparison of sulfur forms for dose 120 mg kg ⁻¹ soil								
no S	10.3	<i>a</i>	11.4	<i>a</i>	8.74	<i>a</i>	10.3	<i>a</i>
S ⁰	7.75	<i>bc</i>	9.18	<i>b</i>	5.49	<i>b</i>	7.81	<i>b</i>
S ⁰ + SO ₄	8.33	<i>b</i>	7.95	<i>b</i>	5.02	<i>b</i>	7.45	<i>b</i>
SO ₄	7.26	<i>c</i>	7.68	<i>b</i>	3.67	<i>c</i>	6.51	<i>c</i>
Comparison of sulfur doses: 60 and 120 mg kg ⁻¹ soil								
no S ⁰	10.3	<i>a</i>	11.4	<i>a</i>	8.74	<i>a</i>	10.3	<i>a</i>
60 mg kg ⁻¹ soil	8.95	<i>b</i>	8.61	<i>b</i>	5.00	<i>b</i>	7.52	<i>b</i>
120 mg kg ⁻¹ soil	7.79	<i>c</i>	8.01	<i>b</i>	4.51	<i>b</i>	6.77	<i>c</i>

Values labeled with the same letter are not significantly different ($p < 0.05$).

3.4. Soil Properties after Experiment

Fertilization with sulfur in both elemental and sulfate forms significantly decreased the soil reaction as compared to the object where this nutrient was not applied (Table 7). It was also found that the size of the sulfur dose had a significant effect on the decrease in soil reaction. It is often emphasized in the literature that the application of sulfur can affect the acidity of soils, especially when applied at higher doses [47–50].

In our study, sulfur fertilization also contributed to a significant decrease in both soil carbon and nitrogen content. The greatest decrease in soil carbon and nitrogen content was observed when a higher dose of sulfur was applied in its elemental form. The availability of sulfur to plants is related to the transformation of organic matter in the soil, which mainly depends on soil microbial activity. In turn, soil microbial activity is dependent on temperature, moisture, pH, and substrate availability [51–53]. Ikoyi et al. [54] found that short-term sulfate fertilization promotes perennial ryegrass growth by outweighing negative feedback from some of the soil biota, while Magnucka et al. [55] found that the application of sulfur fertilizers with the mineral NPKMg promotes soil fertility due to aggregate stabilization and a decrease in water-soluble organic compounds. Nitrogen mineralization from soil organic matter can occur more rapidly compared to the release of sulfur [56,57].

Table 7. Physical-chemical soil properties after experiment.

Treatments	pH		C _{organic}		N _{total}		S _{total}		S-SO ₄		S-SO ₄ /S _{total}	
	1M KCl dm ⁻³		(g kg ⁻¹ Soil)		(mg kg ⁻¹ Soil)		(mg kg ⁻¹ Soil)		%			
	Sulfur dose 60 mg kg ⁻¹ soil											
no S	6.59	a	8.58	a	0.610	a	94	e	3.99	f	4.23	c
S ⁰	6.47	b	6.83	bc	0.544	abc	106	d	12.7	a	11.9	a
S ⁰ + K ₂ SO ₄	6.48	b	7.27	b	0.522	bcd	136	a	10.4	bc	7.65	b
K ₂ SO ₄	6.43	b	6.86	bc	0.468	d	104	d	6.02	ef	6.35	b
S ⁰ + MgSO ₄	6.43	b	5.84	c	0.568	ab	126	ab	7.65	de	6.06	bc
MgSO ₄	6.48	b	7.83	ab	0.532	bcd	110	cd	7.06	de	6.43	b
S ⁰ + (NH ₄) ₂ SO ₄	6.38	b	6.96	b	0.496	cd	117	bc	11.7	ab	10.1	a
(NH ₄) ₂ SO ₄	6.44	b	7.74	ab	0.554	abc	133	a	8.58	cd	6.47	b
Comparison of sulfur forms for dose 60 mg kg ⁻¹ soil												
no S	6.59	a	8.58	a	0.610	a	94	c	3.99	d	4.23	d
S ⁰	6.47	b	6.83	bc	0.544	ab	106	b	12.7	a	11.9	a
S ⁰ + SO ₄	6.43	b	6.69	c	0.529	b	126	a	9.92	b	7.93	b
SO ₄	6.45	b	7.48	b	0.518	b	116	ab	7.22	c	6.42	c
Sulfur dose 120 mg kg ⁻¹ soil												
no S	6.59	a	8.58	a	0.610	a	94	e	3.99	c	4.23	e
S ⁰	6.37	b	5.39	c	0.466	c	147	cd	23.0	a	15.7	a
S ⁰ + K ₂ SO ₄	6.36	b	7.62	ab	0.518	bc	158	b	21.8	a	13.8	ab
K ₂ SO ₄	6.36	b	7.34	b	0.498	bc	144	d	13.5	b	9.37	cd
S ⁰ + MgSO ₄	6.36	b	6.84	b	0.536	bc	159	ab	19.7	a	12.4	bc
MgSO ₄	6.41	b	7.73	ab	0.526	bc	153	bc	10.7	b	7.00	de
S ⁰ + (NH ₄) ₂ SO ₄	6.40	b	7.20	b	0.500	bc	146	cd	21.4	a	14.6	ab
(NH ₄) ₂ SO ₄	6.37	b	7.57	b	0.556	ab	167	a	10.8	b	6.52	de
Comparison of sulfur forms for dose 120 mg kg ⁻¹ soil												
no S	6.59	a	8.58	a	0.610	a	94	b	3.99	c	4.23	c
S ⁰	6.37	b	5.39	c	0.466	c	147	a	23.0	a	15.7	a
S ⁰ + SO ₄	6.37	b	7.22	b	0.518	bc	154	a	21.0	a	13.6	a
SO ₄	6.38	b	7.55	b	0.527	b	155	a	11.7	b	7.63	b
Comparison of sulfur doses: 60 and 120 mg kg ⁻¹ soil												
no S ⁰	6.59	a	8.58	a	0.610	a	94	c	3.99	c	4.23	c
60 mg kg ⁻¹ soil	6.44	b	7.05	b	0.526	b	119	b	9.15	b	7.78	b
120 mg kg ⁻¹ soil	6.38	c	7.10	b	0.514	b	153	a	17.3	a	11.3	a

Values labeled with the same letter are not significantly different ($p < 0.05$).

Under the influence of sulfur fertilization with all analyzed forms of sulfur, the content of total sulfur and sulfate sulfur in the soil increased significantly compared to the control object. Similar studies comparing sulfur fertilization in the sulfate and elementary form also found similar relationships in the content of this element in soil [49]. This may be due to the fact that sulfur applied in the sulfate form is directly available to plants and a significantly higher overall uptake of sulfur by ryegrass was observed in comparison with objects fertilized with the elementary form. This confirms that fertilization with elemental forms of sulfur has a greater subsequent effect of sulfur delivery from the soil compared to sulfate forms.

Maintaining the optimal soil abundance of available forms of sulfur can have both agronomic and environmental benefits [27].

Increasing doses of sulfur also had a significant effect on the content of this element in the soil. Both lower and higher doses of sulfur were found to have the highest sulfate content in the soil when fertilized with the elemental form of sulfur, indicating a slower release of the available sulfur form into the soil during plant growth. This was also confirmed by the highest proportion of the total sulfate form in the soil when fertilizing with elemental sulfur. The combined use of elemental sulfur and sulfate sulfur also significantly increased the sulfate sulfur content of the soil compared with the sulfate form of sulfur. It is safer to use the elemental form of sulfur at higher sulfur rates applied to the grassland due to sulfate leaching into the soil profile [58].

4. Conclusions

The results indicate that fertilizers that are a mixture of S⁰ and sulfate are as effective as those containing only sulfate in terms of stimulating perennial ryegrass growth. Both applied doses of sulfur were equally effective. Fertilization with all forms of sulfur increased the soil's content of this nutrient, making it possible to compensate for deficiencies of this element in the soil. Economic and environmental considerations indicate that the use of fertilizers that are a mixture of elemental and sulfate sulfur is a good approach in agriculture.

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Review

A Crop of High Nutritional Quality and Health Maintenance Value: The Importance of Tartary Buckwheat Breeding

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Abstract: Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.), originating in the Himalayan area, is cultivated in central Asia and northern, central, and eastern Europe. Tartary buckwheat grain and sprouts are rich in flavonoid metabolites rutin and quercetin. The synthesis of flavonoids in plants is accelerated by UV-B solar radiation to protect the plants against radiation damage. During Tartary buckwheat food processing, a part of rutin is enzymatically converted to quercetin. Rutin and quercetin are able to pass the blood–brain barrier. Studies have investigated the effects of rutin and quercetin on blood flow to the brain, consequently bringing more nutrients and oxygen to the brain, and causing improved brain function. In addition to the impact on blood flow, rutin and quercetin have been shown to have antioxidative properties. The goals of breeding Tartary buckwheat are mainly to maintain and enhance the high nutritional quality. The goals could be reached via the breeding of Tartray buckwheat for larger cotyledons. Other main breeding efforts should be concentrated on the easy husking of the grain, the prevention of seed shattering, and the improvement in growth habits to obtain uniformity in grain ripening and a stable and high yield.

Keywords: *Fagopyrum tataricum*; flavonoids; rutin; quercetin; food; nutrition; breeding

1. Introduction

The place of origin of Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) is the mountain area of the Himalayas. Tartary buckwheat cultivation takes place at high altitudes around the Himalayas, in China, Nepal, Bhutan, India, and Pakistan, as well in some other countries: Korea, Japan, Kazakhstan, Russia, Ukraine, Belarus, Sweden, Poland, Italy, Luxemburg, and Slovenia [1,2] (Figure 1a–d). In Bosnia and Herzegovina, Tartary buckwheat is grown mainly as a mixed crop with common buckwheat (*Fagopyrum esculentum* Moench) (Figure 1d) [1].

Tartary buckwheat seeds are protected by a thick husk and phenolic substances, and may remain alive but dormant in the soil for many years. Under suitable environmental conditions, they can grow again. Tartary buckwheat survives under strong ultraviolet radiation, which takes place at high altitudes. During their evolution, the plants survived while gradually accumulating genes for substances that allow the plants to reproduce and survive in the stony areas of the high Himalayas, exposed to intense UV-B radiation. The main protective substances that will be discussed in the present paper are flavonoids and other phenolic substances. There are many steps in the synthesis of flavonoid substances and phenolic acids based on phenylalanine; synthesis is enabled by genes and enzymes, active under the impact of UV-B radiation (Figure 2) [2]. The important function of Tartary buckwheat phenolic substances is thus the protection of plants against UV-B radiation.

Many genes involved in the biosynthesis of phenolic substances and their regulation were established in buckwheat [3]. The feasibility of Tartary buckwheat to be able to survive in conditions of high levels of abiotic stress is attributed to the development of

several complexes of genes influencing the transduction of signals and the regulation of genes [3]. Such changes in the genetic layout cause the adaptation of plants to harsh ecological conditions. The significance of the genes for the biosynthetic pathway of rutin and the relevant MYB transcription factors (Figure 2) [3–5] is well-known.



Figure 1. (a) Flowering and ripening plant of Tartary buckwheat. (b) Inflorescence with ripening Tartary buckwheat grain. (c) Field of Tartary buckwheat in Wermland, Sweden. (d) Fields growing a mixture of Tartary and common buckwheat in the central part of Bosnia and Herzegovina.

Buckwheat metabolites with benzene rings included protect buckwheat plants in several ways. The protection of buckwheat plants from fungal attacks is important for fungi that form mycotoxins in the grain [6–8]. In food, however, the plant-protecting substances of Tartary buckwheat are important for protecting human health.

The flavonoid rutin is present in the grain of Tartary buckwheat, mainly in the cotyledons [9–11]. In addition to rutin, there are also enzymes in the grain which transform rutin into quercetin (Figure 3). Rutin and quercetin molecules are similar, with the difference that rutin has two sugar molecules attached to the aglycone part of the molecule. The enzyme rutinase enables the splitting apart of the sugar part of the aglycone and the transformation of rutin into quercetin. In the intact grain, rutin is separated from the enzyme and thus is protected from the transformation. After crushing Tartary buckwheat seeds, the rutin-degrading enzyme is mixed with rutin and during the preparation of dough, after moistening, the conditions are suitable for the decomposition of rutin molecules and the appearance of quercetin. As the consequence, the bitter substance quercetin appears [12–14].

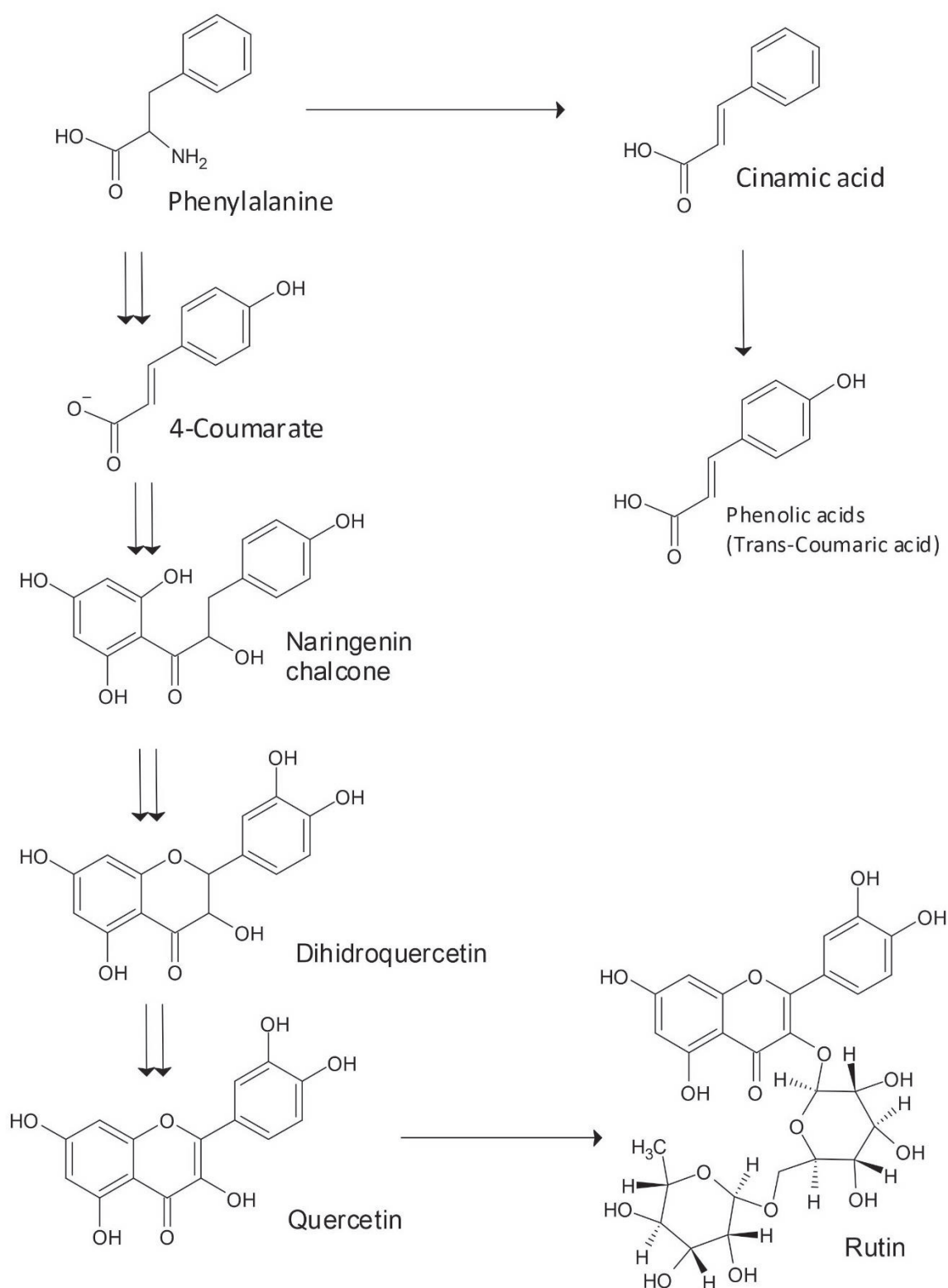


Figure 2. Main steps in the biosynthesis of rutin from phenylalanine in buckwheat plants, each supported by respective genes. A side branch of the synthetic pathway leads to phenolic acids.

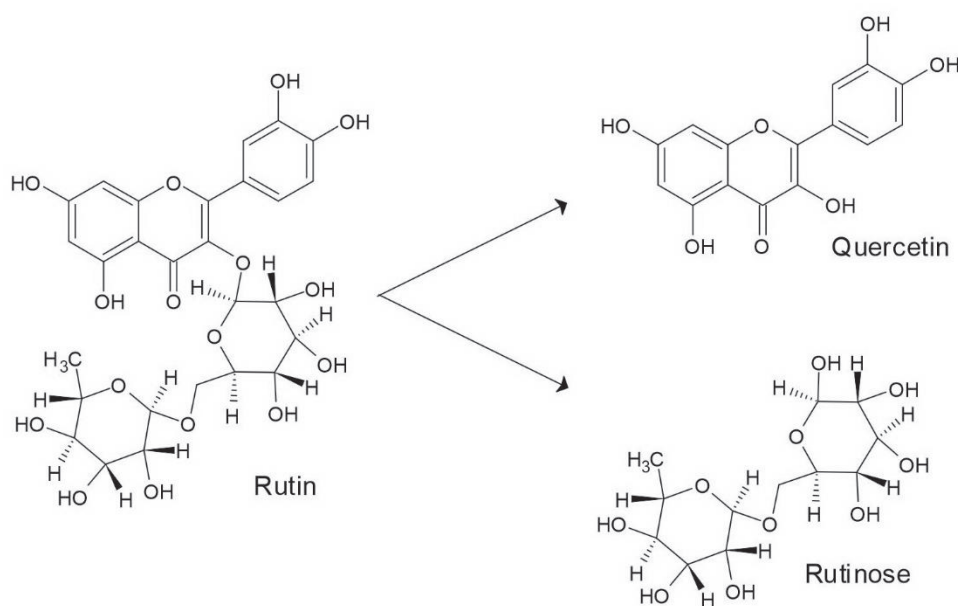


Figure 3. The enzymatic degradation of rutin to flavonoid quercetin and sugar rutinose.

Tartary buckwheat has, in comparison to cereals, a high nutritional value. It has good fatty acid composition and excellently balanced amino acid composition, with favorable vitamin B content [15–17], and a very high rutin level [18–20]. Rutin is known for its ability to strengthen blood vessels, supporting the impact of vitamin C and aiding in reducing blood clots and cholesterol levels [21–23]. Consuming dishes with Tartary buckwheat is important for the prevention of gallstone formation, hypertension, cardiovascular problems, and obesity [24–26]. The effects are mainly due to slowly digestible proteins, digestion-resistant starch, phenolic substances, and their interactions with other grain constituents [27]. From a comparison of the amounts of flavonoids rutin and quercetin in the collection of Tartary buckwheat grain and sprout samples from Europe and Asia, it was reported that the Nepal grain samples had the highest rutin content (13.3 g/kg in dry matter). The concentration of rutin in the sprouts from the same Himalayan area was up to 54.4 g/kg in dry matter. The concentration of quercetin in sprouts was 10–90-fold higher in comparison to that established in the grain [27,28]. Tartary buckwheat grain and sprouts have important potential for providing flavonoids in the human diet and for the production of functional dishes, rich in flavonoids.

When comparing common buckwheat and Tartary buckwheat, there is a considerable difference in the metabolic layout of the two species. It was found that 61 flavonoids and 94 non-flavonoid substances had significantly higher content (at least double) in Tartary buckwheat in comparison to common buckwheat. Tartary buckwheat flour has a yellow color, compared to the light gray color of common buckwheat flour (Figure 4a). The dough and bread made from Tartary buckwheat flour also express a yellow-green hue (Figure 4b–d). Tartary and common buckwheat grains are rich in secondary metabolites beneficial to human health and among them, non-flavonoid metabolites are important. They contribute high health-supporting effects in Tartary buckwheat compared to common buckwheat [29,30]. Zhang et al. [31] reported the sequencing and assembly of Tartary buckwheat [31–33]. Milling the grain of Tartary buckwheat and mixing the flour with water results in the formation of quercetin, which is a degradation product of rutin after rutinase activity [14,32,33].



Figure 4. (a) Tartary buckwheat flour (left) compared to common buckwheat flour (right) at the farmers' market in Xichang, Liangshan Yi Autonomous Prefecture, Sichuan, China. (b) Dough made from Tartary buckwheat flour, expressing a yellow-green hue due to Tartary buckwheat's secondary metabolites. (c) Pretzels made from a mixture of Tartary buckwheat (40%) and wheat flour, expressing a green hue due to the Tartary buckwheat's secondary metabolites content. (d) Slovenian olive-oil-seasoned vegan soup, thickened with boiled husked common buckwheat grain (to the left) and husked Tartary buckwheat grain.

Many novel dishes have recently been developed based on traditional Tartary and common buckwheat grain materials, rich in rutin and quercetin (Figure 4c,d) [11,34,35]. Due to the protein content, with suitable amino acid composition, innovative foods based on Tartary buckwheat are a source of high quality proteins in the diet of vegans and other people who do not consume animal proteins. In Korea and China, Tartary buckwheat sprouts are developed as a food material, rich in flavonoids rutin and quercetin [36].

2. Bioactivity of Flavonoids Rutin and Quercetin

In Tartary buckwheat, the complexation of quercetin with starch has been reported [37]. Enzyme molecules hinder the *in vitro* digestion of starch complexed with quercetin [37,38]. This causes the transformation of native starch to the resistant one and changes the physical and chemical characteristics of the Tartary buckwheat starch [39]. The effects of such a quercetin–starch complexation are that foods made from Tartary buckwheat will have limited digestibility. Quercetin in Tartary buckwheat can reduce the concentration of low-density lipoprotein, serum triacylglycerols, and body weight. In experimental animals, a diet with 0.1% quercetin was shown to significantly impact the lowering of low-density lipoprotein concentrations in serum, without any effect on high-density lipoprotein. Tartary buckwheat has also prevented an increase in body weight and fat deposition during high-

fat intake in rats [40]. A buckwheat diet can improve glucose intolerance in patients by reducing the insulin response [41]. The reduced postprandial glycemic responses in common buckwheat were shown [41].

Phenolic substances are often transformed in the gut before their absorption. The microbiota in the colon enable this process [42]. Small-sized phenolic molecules, which result from the colon's microbial conversion, are more easily absorbed than large-sized phenolic metabolites [43–45].

Radiation-induced brain injury is a serious adverse effect of brain radiotherapy in oncology. Flavonoid quercetin has a wide range of biological activities, including the ability to regulate gut microbiota. After the oral submission of quercetin preparations in experimental mice, the spontaneous activity behavior, short-term memory ability, and anxiety level were improved [46]. The long-term administration of buckwheat whole meal flour suppresses cognitive decline by increasing hippocampal brain-derived neurotrophic factor production in experimental mice [47].

Important bioactivities have been found for quercetin and the related molecules, not just in the gastrointestinal system but also in blood vessels, muscles, and the brain [48]. The blood–brain barrier is a highly selective semipermeable border, consisting of endothelial cells. It prevents solutes from circulating blood from non-selectively crossing into the extracellular space of the central nervous system [49–51]. Ingested rutin and quercetin can accumulate in the brain tissue after crossing the blood–brain barrier [52]. The challenge in treating Alzheimer's disease and some other neurological conditions is the inability of medical substances to enter the brain due to poor solubility and the blood–brain barrier [53]. Quercetin could cross the blood–brain barrier and exerts neuroprotective effects in many neurological disease situations [54]. In vitro and in vivo experiments have shown that molecules, including quercetin, and quercetin derivatives, can penetrate the blood–brain barrier without toxicity; quercetin could be delivered to the ischemic area of the brain, ensuring the targeted delivery and antioxidative impact. The mitochondrial targeting of damaged neurons is also achieved in such a way. Substances with quercetin as part of the molecules may have better neuroprotective ability than quercetin itself [55].

Taile et al. [56] established that polyphenols are important dietary antioxidants with anti-inflammatory action, able to improve the effects of stroke and other cerebrovascular problems. The protective impact of quercetin and some other polyphenolic substances is connected with their bioavailability. It is important to apply polyphenol-based strategies for the improvement of the clinical picture of stroke.

Blood–brain barriers hinder not only the endogenous or exogenous toxicants, but also compounds with therapeutic properties. A group of chemo-sensing receptors was identified in the blood–cerebrospinal fluid barrier. Chemosensing bitter taste receptors are promising as potential targets of drugs. Quercetin, resveratrol and other metabolites with neuroprotective activity as ligands for transporting molecules can potentially counteract drug resistance in the delivery to the brain for the treatment of central nervous system disorders [57,58]. Extracts from buckwheat leaves and flowers have an impact on the antioxidant situation of the liver and brain of mice. Such extracts were given orally to mice for 21 days. The effects in the mouse brain were the following: the amount of glutathione and malondialdehyde was reduced, superoxide dismutase activity was significantly decreased, and catalase activity significantly increased [59].

Choi et al. [60] studied the impact of rutin and the n-butanol fraction extracted from Tartary buckwheat on learning and memory deficits in a mouse model of amyloid beta (A beta)-induced Alzheimer's disease. The impaired cognition and memory of experimental animals were attenuated by the oral submission of an n-butanol fraction and rutin extracted from plants of Tartary buckwheat. According to the results from Choi et al. [60], the n-butanol fraction and rutin extracted from Tartary buckwheat had protective effects, and therapeutic applications for the treatment of Alzheimer's disease are thus suggested.

3. Precautions for Possible Adverse Effects of Tartary Buckwheat Metabolites

One of buckwheat's secondary metabolites, fagopyrin, has a health threat when the green parts of common or Tartary buckwheat are eaten [61–63]. But the ingestion of buckwheat grain products seems to be safe due to the low concentration of fagopyrin in the grain [63–65]. A buckwheat allergy is a very rare event [66].

Suzuki et al. [12] investigated the potential toxicity of rutin-rich dough made from Tartary buckwheat through acute and subacute toxicity studies in experiments with laboratory rats. The concentration of rutin in Tartary buckwheat material was 1570 mg/100 g. In the experiment, no toxic or other non-regular symptoms were detected. The resulting body weight was not significantly different among the groups of experimental animals. The conclusion of the experiment was that Tartary buckwheat at a given dose was without noticeable adverse effects. The results of Vogrinčič et al. [67] and Suzuki et al. [68] also established that no genotoxic effects were expressed in Tartary buckwheat grain materials.

Quercetin is genotoxic to salmonella, but it was approved to be safe for human application [69]. The study of Cunningham et al. [70] confirmed the safety of quercetin for use in mice when applied daily at 12.5, 25, or 50 mg/kg of body weight for 98 days. In any case, more studies have to be performed to determine any possible quercetin toxicity effects after the chronic ingestion of Tartary buckwheat or other quercetin-rich food [70].

4. Main Goals and Methods for Breeding Tartary Buckwheat

Tartary buckwheat is a semi-wild plant [1]. It was not long ago when people started to cultivate it. Even now, wild forms of Tartary buckwheat grow outside the fields on the rocky slopes of the Himalayas. Tartary buckwheat is found as a weed in common buckwheat crops or other crops. The shattered seeds may return from the fields to less friendly areas, as wild plants, occupying poorly fertile soil. For the propagation of wild plants, the shattering is a very important trait. In newly domesticated crop-like Tartary buckwheat, seed shattering is a detrimental characteristic for growing in fields and consistent efforts must be made to eliminate this trait during breeding. When Tartary buckwheat is cultivated, an important part of the yield could be lost by the shattering of seeds. Therefore, breeding against shattering and obtaining the uniform ripening of Tartary buckwheat seeds are important goals in Tartary buckwheat breeding. In practical terms, the selection against shattering could be performed by selecting thicker peduncles supporting individual seeds. Other selection methods against seed shattering could be through the vibrating of plants and scoring which portion of seeds remain on the plants after the vibration impact. This selection method is not very effective because of the non-uniform ripening of the seeds on Tartary buckwheat plants. An important issue in obtaining less shattering is a uniform time for ripening of all seeds on the plant. In common buckwheat, the gene for the determinate growth habit is known [71–73]. Determinate buckwheat plants have more uniform ripening and stronger side branches, which supports the prevention of seed shattering. But until now, no report on determinate growth habits in Tartary buckwheat is known. It should be desirable to find Tartary buckwheat with determinate growth habits. One way to reach this goal could be a mass screening of the Tartary buckwheat population for this trait, or mutation breeding. Determinate common buckwheat plants are lower, so they could be hidden in the canopy. Determinate plants in the tight canopy condition have a lower possibility of surviving and reproducing seeds. Therefore, in canopies, selection pressure is working against plants with determinate growth habits, unless plants grow in conditions with less competition from neighboring plants [71,74,75]. Why determinate Tartary buckwheat plants have not yet been found among the wildy growing populations is unknown. Determinate Tartary buckwheat would be interesting because of its expected resistance to lodging, the simultaneous and uniform seed set and the resistance to seed shattering. One of the main problems with the production of Tartary buckwheat is its indeterminant growth. As a result, the seeds ripen very unevenly and the first ripe seeds can fall off before the last formed and filled seeds mature.

Mutation breeding in Tartary buckwheat should not be too complicated as it is a self-fertile plant and homozygotes for the mutated gene could already be expressed and scored in early generations after treatment, with mutagenic impact.

The lodging of plants does not seem to be a serious problem in Tartary buckwheat. If lodged, plants are very soon recovered. If plants can be created by breeding the determinate Tartary buckwheat, they are expected to be more resistant to lodging than non-determinate plants. For example, in common buckwheat, determinate plants are much better resistant to lodging in comparison to non-determinate plants [71].

As Tartary buckwheat grains have coarse and hard husks, it is very difficult to dehusk them to obtain groats. Only a few producers can make husked Tartary buckwheat [1]. Several easily husked or “Rice-Tartary” buckwheat forms are known. Rice-Tartary is a special type of Tartary buckwheat, with seeds with a loose husk that make dehusking easier [76,77]. It was established that by reciprocally crossing Tartary and rice-Tartary buckwheats, and backcrossing rice-Tartary-type progeny with Tartary buckwheat, the non-adhering husk trait depends on a single recessive gene [78]. However, according to the new results from Duan et al. [79], three genes regulate the trait of easily husked Tartary buckwheat.

In crossing, hot water emasculation was used. The hybrid rice-Tartary buckwheat cultivar Mikuqiao18 was obtained in China through the pedigree selection of crossbreeding ‘Miqiao’ with ‘Jingqiaomai2’ [77]. The hybrid had a lower yield than parent varieties because the grain mass was lower due to the thinner husk.

In Tartary buckwheat grain, the most valuable substances for good nutritional value and the maintenance of human health are cotyledons. The increased size of cotyledons is important for a high content of proteins and polyphenols. It is not yet clear how to select plants to achieve this goal. One possibility would be to evaluate the size of cotyledons through the transverse sections of grain. Another possibility would be to estimate the size of cotyledons through the evaluation of milling fractions. Contrary to the situation in common buckwheat, in Tartary buckwheat all the grain on a given Tartary buckwheat plant is genetically uniform because of self-fertilization. So, a certain grain from a Tartary buckwheat plant would be, genetically, a representative sample for other seeds from the same plant.

It has been established that the expression levels of genes for phenylalanine ammonia lyase (PAL) and 4-coumaric acid coenzyme A ligase (4CL) were positively correlated with the content of flavonoids in Tartary buckwheat [80]. This finding suggests the possibility for breeding Tartary buckwheat with stable or enhanced flavonoid content, which is very important for the utilization value of Tartary buckwheat.

Other important quality traits are plant height, leaf blade width, stem color (green or red), number of primary branches, inflorescence length, flower color (greenish-yellow, white, pink, red), seed anthocyanin color (green, brown, black), seed shape (ovate, with sharp edges or with growths) and seed weight. These accessions can be of vital significance for future buckwheat breeding programs.

Traditional breeding and selection methods are chiefly concentrated on visible properties, including plant morphology and yield. The changes in metabolic profile cannot be observed by the methods usually used. Research on changes in the metabolite profile during domestication and breeding efforts is very important. Metabolome profiling in Tartary buckwheat will make genetic improvements of traits possible in Tartary buckwheat, which is important for medical use and resistance against diseases [81].

Tartary buckwheat is mainly tolerant to pests and plant diseases. In any case, strains of *Rhizoctonia solani*, a soil-born pathogen, may damage the plants [82]. Using a multiomic approach, it is feasible to identify genes related to resistance against this pathogen in Tartary buckwheat. This finding could accelerate the molecular breeding of *Rhizoctonia*-resistant cultivars in Tartary buckwheat [82]. Regarding climatic changes, obtaining Tartary buckwheat cultivars that are tolerant to drought is essential. Relevant genes and regulatory systems connected to drought tolerance have been reported in Tartary buckwheat [83].

The transformation and genome editing of common and Tartary buckwheat have some restrictions in their application [84]. In several European countries (for example, Austria, Czech Republic, and Italy), common buckwheat and Tartary buckwheat are grown and used in nutrition as ecological crops. According to the official demands for ecological crops, the use of transgenic cultivars is not allowed. Moreover, transgenic cultivars should not be cultivated close to ecological crops, to prevent the pollination of ecologically grown plants with the pollen of transgenic plants [84].

5. Future Perspectives

Due to its excellent nutritional value and its adaptability to adverse climatic and soil conditions, Tartary buckwheat undoubtedly has a future. Above all, it is necessary to increase the yield. Care must be taken to ensure that entire yield is harvested on the fields, i.e., it is necessary to improve the resistance of Tartary buckwheat to weather incidents, to prevent lodging and losing seeds through shattering. The starting point for the successful breeding of Tartary buckwheat is genetic material that must be collected and used in breeding.

The genetic material of Tartary buckwheat is available from diverse sources. The starting material for breeding could be the domestic populations of Tartary buckwheat grown by farmers, especially in areas of the Himalayas. The starting genotypes for successful Tartary buckwheat breeding could also be wild plants, and weedy Tartary buckwheat plants growing in the fields among the plants of common buckwheat. Weedy Tartary buckwheat plants are already adapted to grow in cultivated fields. Within Tartary buckwheat populations there is a great diversity and polymorphism, so selection could be very effective.

To increase the diversity and accelerate breeding, induced mutations are feasible, as well as obtaining tetraploids or crossing Tartary buckwheat plants with plants of other, related species.

When breeding for increased yield, care must be taken to maintain and improve the resistance of plants against diseases, pests and weather disasters. Care should also be taken to preserve the excellent nutritional value of Tartary buckwheat, especially its high content of flavonoids, other polyphenolic substances, proteins and fiber.

6. Conclusions

Tartary buckwheat grain and sprouts are rich in flavonoids rutin and quercetin. After crushing Tartary buckwheat grain and mixing the obtained material with water, the rutin-degrading enzyme of the grain starts its activity of decomposing the rutin molecules, as the result the concentration of rutin becomes lower and the concentration of quercetin becomes higher.

Studies have shown the effects of flavonoids rutin and quercetin on the human body, including the brain, producing promising results. Rutin and quercetin have been shown to have anti-inflammatory effects, which are believed to play a role in various neurological conditions, including depression, anxiety, and Alzheimer's disease. Despite the promising results, further research is needed to completely understand the effects of rutin and quercetin and to determine the optimal dose for long-term use. Tartary buckwheat is a valuable food source of flavonoids rutin and quercetin and other phenolic metabolites. However, maintaining a balanced diet is most important, and relying on a single food as the main source of nutrients should be avoided.

Tartary buckwheat is a self-fertile plant, so mutation breeding is feasible. Few generations after mutagenic treatment, homozygotes for mutated genes could appear and mutated traits would be visible for selection. Another breeding method is screening Tartary buckwheat varieties and wild populations for desirable traits, and using hybridization to introduce the desirable traits to the target cultivars.

The main goal of Tartary buckwheat breeding is suggested to be maintaining and enhancing the excellent properties of flavonoid content and the high nutritional value of proteins.

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