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Article

Relationships Between Photosynthetic Efficiency and Grain Antioxidant Content of Barley Genotypes Under Increasing Nitrogen Rates

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Abstract: Nitrogen fertilization may affect the functioning of photosynthesis as well as the chemical composition and antioxidant potential of cereal grains. Little is known about the relationship between the efficiency of photosynthesis and the content of phenolic compounds in barley grain, especially in conditions of varying nitrogen availability. In this regard, a field experiment was conducted to examine the responses of two primary barley genotypes with elevated phenolic compound content (TPC) in grain and an intensive modern cultivar *H. v. vulgare* with high protein content to increasing nitrogen fertilization (rates of 0, 30, 60 and 90 kg N ha^{-1}) during the study years, which differed in terms of hydrothermal conditions. The leaf greenness index (SPAD) and chlorophyll fluorescence parameters were evaluated on three occasions throughout the growing season. Following the harvest, the chemical composition of the grains, including phenolic acids, flavonoids and antioxidant potential, was evaluated. The antioxidant potential and chemical composition of the grain, including TPC and protein content, depended to the greatest extent on genetic and environmental factors, and only then on nitrogen fertilization. Nitrogen increased the TPC content and antioxidant capacity ABTS$^+$ of the grains of all studied genotypes and the protein content in *H. v. vulgare* grain. Rates of 60 and 90 kg N ha^{-1} resulted in a significant increase in the SPAD, PI_{abs} and F_v/F_m in BBCH 34 and 57. A positive correlation was confirmed between the SPAD and PI_{abs} and the content of TPC and ABTS$^+$ in the grain. The dependence of qualitative characteristics on the F_v/F_m was also demonstrated. The primary genotypes are characterized by a greater genetic potential for the synthesis of phenolic compounds than the modern cultivar *H. v. vulgare*. The synthesis of phenolic compounds, and thus their accumulation in the grain, is clearly stimulated by unfavorable environmental factors and moderate nitrogen rates and depends on the chlorophyll content in the leaves and the efficiency of photosynthesis. N fertilization has a beneficial effect on the content of phenolic compounds in grain resulting from the improvement in the SPAD and PI_{abs}. The chemical composition of grain and the increase in antioxidant potential are determined by the F_v/F_m, which is low under hydrothermal stress conditions.

Keywords: phenolic compounds; cereals; green leaf index; chlorophyll fluorescence; N fertilization; *Hordeum vulgare* L.

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1. Introduction

Nitrogen fertilization is one of the basic factors stimulating plant productivity and fertility. It is estimated that in the last century, the application of artificial fertilizers contributed approximately 50% to the overall increase in agricultural crop yields, which explains their widespread use [1]. However, the use of nitrogen fertilizers has contributed to the overall deterioration of the natural environment [2]. It may also disturb the proper chemical composition and nutritional value of agricultural produce [3].

For many years, plant breeding has also focused on increasing yields by creating varieties that respond well to the intensification of cultivation, including fertilization [4]. Moreover, it has been important to obtain forms that in their main yield concentrate large amounts of nutrients important for the further use of raw materials, such as protein, gluten and starch. Obtaining high-yielding cultivars with high concentrations of basic nutrients has been achieved, however, at the cost of losing many health-promoting compounds [5]. In this context, a lot of research has recently appeared on primary forms of cereals which, due to their primary nature, do not require such large doses of nutrients or pesticides [6] and are an excellent source of bioactive compounds [7,8].

The low content of bioactive compounds consumed in agricultural products may be one of the main causes of the increasing incidence of diet-related diseases such as coronary heart disease, diabetes, Parkinson's disease and even cancer [9,10]. One of the most important groups of compounds with proven health-promoting properties is phenolic compounds. In addition to the positive impact of phenols on human and animal health, they play a very important role in the physiology of plants, reducing biotic and abiotic stresses to which plants are subjected during growth [11]. Phenolic compounds are involved in immune mechanisms in the case of, among others, the attack of pathogens [12], pests, the toxic effects of heavy metals [13] or cultivation under unfavorable environmental conditions [14]. Biotic and abiotic stresses are the primary stresses that plants experience, and these can give rise to secondary stresses that are related to the production of reactive oxygen species (ROS). An imbalance between the amount of ROS in the plant cell and the efficiency of their neutralization results in oxidative stress, which causes damage to nucleic acids, proteins, carbohydrates, lipids and plant pigments such as chlorophyll. In order to maintain the normal course of vital functions, the plant regulates ROS levels through enzymatic and non-enzymatic systems. The latter system is mainly based on a large group of phenolic metabolites, some of which, such as salicylic acid (SA), also function in the plant as phenolic plant hormones, signaling and regulating numerous plant responses to biotic and abiotic stresses [11]. Their concentration in the plant, in addition to the mentioned stresses and genotypic traits, may also be determined by cultivation technology [15]. According to Barański et al. [16], the level of nitrogen nutrition is of crucial importance in shaping the content of phenolic compounds in plants. In the literature, we can find examples indicating the stimulating effect of mineral nitrogen on the concentration of phenolic compounds [17,18]. On the other hand, some studies report that the deficiency of this macroelement intensifies the production of phenols in plants, as compounds responsible for many defense reactions in their tissues [19,20]. According to Sun et al. [21], nitrogen fertilization significantly increases the biomass and N content in the plant but reduces the concentration of phenolic compounds in a rate-dependent manner, which is associated with the inhibition of phenylalanine ammonia lyase and the expression of key genes. As emphasized by Falcinelli et al. [20], the deficiency of basic minerals is a highly stressful factor for plants that can disrupt the most basic life processes of plants. The photosynthetic activity of leaves is subject to influence from a range of environmental and agrotechnical factors. This is related to a number of biochemical reactions, enzymatic activities and gene expressions observed in plants [22]. According to Szczepanek et al. [23], the efficiency of photosynthesis varies depending on the chlorophyll content in the leaves, which depends on the interaction of growing conditions and genotype. Different genotypes of the same species may show different reactions to the same environmental and agrotechnical factors [24].

To sum up, nitrogen fertilization may have a significant effect on the content of phenolic compounds in cereal grains. However, the available literature does not clearly indicate the direction of these changes. There is also a lack of research on the relationship between the physiological state of plants and the content of phenolic compounds in barley grain.

The research hypothesis was that nitrogen fertilization would have a significant effect on plant physiological parameters and the content of phenolic compounds in barley grain. It was hypothesized that the observed responses would vary depending on the specific genotype and the hydrothermal conditions prevailing during the growing season. It was hypothesized that the availability of nitrogen would have a greater effect on the content of phenolic compounds in the original genotypes with black grain, while the modern variety would respond with an increase in protein content. Furthermore, it was postulated that suboptimal hydrothermal conditions would diminish photosynthetic efficiency, thereby prompting the accumulation of phenolic compounds and antioxidant potential in barley grain.

The aim of the study was to assess the relationship between the physiological state of barley plants and the content of phenolic compounds as well as the antioxidant activity of grain under conditions of increasing rates of mineral nitrogen fertilization. Two original black grain barley genotypes with naturally elevated concentrations of phenolic compounds in the grain were compared with a modern variety as a reference trial in two growing seasons different in terms of hydrothermal conditions.

2. Materials and Methods

2.1. Plant Material

The study material consisted of three barley genotypes. Two of them were the original genotypes *Hordeum vulgare* L. var. *nigricans* (Ser.) Körn and *H. vulgare* L. var. *rimpaui* Wittm. These barleys are two-row spring barleys and are characterized by black grain color. The third is the modern intensive cultivar 'Soldo' *Hordeum vulgare* var. *vulgare*. It is a two-row spring variety with yellow grain. *H. v. rimpaui* is also distinguished by its reduced awns, the so-called hooded barley, while *H. v. nigricans* and *H. vulgare* have ears with awns.

2.2. Agronomic Practice and Site Description

A two-year field experiment was conducted in Minikowo, Kuyavian–Pomeranian Voivodeship, central Poland (53°1,000,200 N, 17°4,402,200 E) in 2021–2022 in a split-plot design in four replicates on 24 m^2 plots. The first-order factor was the diverse barley genotypes at three levels: *H. v. rimpaui*, *H. v. nigricans* and *H. v. vulgare*. The second-order factor was fertilization at rates of 0, 30, 60 and 90 kg N ha^{-1}. Barley was sown in the third 10-day period of March at a row spacing of 12.5 cm and a density of 350 grains m^{-2}. Grain was harvested at 14% moisture content, in both years between 21 and 31 July.

The characteristics of the chemical composition of the soil are shown in Table 1. The content of available forms of potassium (K) and phosphorus (P) was determined using the Egner–Riehm (DL) method, while the content of available magnesium (Mg) was determined using the Schachtschabel method. The content of C_{org} was determined using the Vario Max CN analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany). The weather conditions in the barley growing season during the experiment were characterized by large differences between the study years (Table 2). In 2021, significantly lower temperatures were observed in the period from 21 April to 10 May, while higher temperatures were observed in the periods from 1 to 20 June and 1 to 20 July than in 2022. In the 2nd and 3rd ten-day periods of April in 2021, coinciding with emergence, frosts were observed, with temperatures at ground level sometimes falling to −7.0 °C. In 2022, during the same period, temperature drops ranged from −2.1 to 6.2 °C. The total precipitation during the barley growing season (March to July) in 2021 was 208.5 mm and in 2022 only 131.7 mm. A pronounced rainfall deficit was particularly characteristic of the period from 21 April to 31 May 2022, with a rainfall total of only 25.4 mm. At the same time in 2021, rainfall was 75.5 mm.

Table 1. Chemical composition of the soil of the fields in the 2021 and 2022 experiments.

Year	N_{min}	P_2O_5	K_2O	Mg	pH	C_{org}
	(kg ha^{-1})		(mg 100 g^{-1} of Soil)		(KCl)	(%)
2021	27.3	21.1	16.7	5.3	5.5	0.10
2022	20.9	17.4	11.4	3.9	5.1	0.06

Table 2. Weather conditions in the barley growing seasons in the years 2021–2022 at the experimental site.

Period	T_{avg} (°C)		T_{max} (°C)		T_{min} (°C)		$T_{min\ at\ ground}$ (°C)		Precipitation (mm)		Sielianinov Index	
	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022
1–10 April	4.0	3.9	16.9	15.7	−3.5	−6.0	−10.2	−9.6	4.8	10.5	1.2	2.7
11–20 April	7.1	6.9	19.3	19.0	0.3	−2.6	−5.3	−7.3	20.3	13.2	2.8	1.9
21–30 April	6.4	9.5	15.7	16.9	−3.3	1.0	−7.0	−3.2	8.1	0.6	1.3	0.1
1–10 May	8.6	11.8	25.3	21.1	0.8	3.2	−1.0	−1.0	26.6	2.2	3.1	0.2
11–20 May	14.5	14.3	27.2	25.5	4.1	−1.4	0.3	−6.2	11.1	6.7	2.3	0.5
21–31 May	11.6	12.5	19.3	20.6	4.9	3.8	1.9	−1.2	29.7	15.9	0.5	1.2
1–10 June	17.6	16.2	26.0	24.7	7.2	5.3	3.5	1.2	8.8	8.5	0.8	0.5
11–20 June	19.4	17.8	30.7	34.6	7.3	7.1	3.7	3.0	8.9	23.5	0.5	1.3
21–30 June	20.4	20.7	31.2	31.4	10.5	3.4	6.9	5.0	23.1	7.3	1.1	0.4

T_{avg}—average air temperature; T_{max}—maximal air temperature; T_{min}—minimal air temperature; $T_{min\ at\ ground}$—minimal air temperature at ground; precipitation—sum of precipitation; Sielianinov index—hydrothermal condition index of Sielianinov.

The hydrothermal conditions (Table 1) observed during the study years were also described using the Sielianinov coefficient [25]; the classification given in Kuklik et al. [26] was used to assess the coefficient value.

2.3. Leaf Greenness Index (SPAD) and Chlorophyll Fluorescence

In the second leaf stage (BBCH 12), according to the Biologishe Bundesanstalt Bundessortenamt and CHemische Industrie scale, in the stem elongation stage (BBCH 34) and ear development (BBCH 57), the SPAD index was determined on the 30 youngest fully developed leaves in 4 replicates for each subject, using a Minolta N-tester (Konica-Minolta, Tokyo, Japan). Direct chlorophyll a fluorescence was also measured in barley plants at the same developmental stages. The tests were carried out using a Pocket PEA (Pocket Plant Efficiency Analyzer) fluorimeter (Hansatech Instruments, Pentney, UK). Chlorophyll fluorescence measurements were carried out on 4 mm^2 samples from the central part of the mature leaf blade, from the youngest fully developed leaf, i.e., from the first leaf at BBCH 12, from the subflagellated leaf at BBCH 34 and from the flag leaf at BBCH 57. The light phase of photosynthesis was extinguished using special clips that blocked the light entering the sample 30 min before the measurement. The measurement was performed according to the instrument manufacturer's recommendations, with a light pulse intensity of 3500 µmol-m^{-2}-s^{-1} and a duration of 1 s. The parameters Fv/Fm—the maximum yield of PSII—and PIabs—an indicator of the functioning of PSII in relation to absorption—were assessed.

2.4. Determination of DPPH and ABTS$^+$ Radical Scavenging Capacity

The radical scavenging capacity was assessed using the adapted 2,2′-diphenyl-b-picrylhydrazyl (DPPH) method described by Zhou et al. [27]. For this purpose, the extract (10 µL) was added to 195 µL of ethanolic DPPH solution (120 µM). The reaction mixture was pipetted into 96-well plates and incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm.

Free radical scavenging using the stable ABTS$^+$ radical was performed according to a modification of the improved ABTS$^+$ method described by Zhou et al. [27]. The determination was carried out in 96-well plates. The extract (10 µL) or ethanol (10 µL, control) was added to 195 µL of ABTS radical solution and allowed to stand for 30 min until a stable absorbance reading was achieved. The decrease in absorbance at 734 nm was measured against the blank (ethanol).

2.5. Determination of Total Phenolic Compounds (TPC)

The total polyphenol content of the extracts obtained was assessed using a colorimetric method according to the methodology given by Ventura et al. [28]. The method was adapted to microplate assays and adapted to small volumes, viz: 20 µL of the extract (1 mg/mL) was mixed with 20 µL of Folin–Ciocâlteu reagent (Sigma-Aldrich, Poznań, Poland) in the well. After 5 min, 20 µL of sodium carbonate (0.01 M) was added to each sample and allowed to stand for 5 min. The solution was then diluted in 125 µL of distilled water and the absorbance of the distilled water was read with a spectrophotometer (Epoch, BioTek Instruments, Inc.; Winooski, VT, USA) using Gen5 Data Analysis software. A wavelength of 790 nm was used for the study. Measurement results were expressed in milligrams of gallic acid equivalents per liter of extract (mg GAE/L) according to the GA standard curve. The maximum absorption wavelength was determined in the UV–Vis range of the GA standard (Sigma-Aldrich, Poznań, Poland) solution. A wavelength of 790 nm was used for the study.

2.6. Determination of Phenolic Acids

The content of the phenolic acids gallic acid, 2,5-dihydroxybenzoic acid, 4-dihydroxybenzoic acid, protocatechuic acid, syringic acid, p-coumaric acid, chlorogenic acid, caffeic acid, synaptic acid and ferulic acid was determined according to the method described in the paper by Nowak et al. [29]. Ground 0.2 g grain samples were subjected to alkaline hydrolysis in 4 mL of 2M sodium hydroxide aqueous solution, followed by acid hydrolysis in 2 mL of 6M hydrochloric acid aqueous solution. The phenolic acids were extracted from the inorganic phase with diethyl ether (2 × 2 mL). This was followed by the acid hydrolysis of the ether extracts in 3 mL of 6M aqueous hydrochloric acid solution. The resulting ether extracts were evaporated to dryness in a stream of nitrogen and then dissolved in 1 mL of methanol. Chromatographic analysis was performed using a Waters SDS 501 high-performance liquid chromatograph (Waters, Milford, MA, USA) with a Waters 486 Tunable Absorbance Detector (Waters, Milford, MA, USA). Chromatographic separation was performed on an RP C-18, 250 × 4 mm × 5 µm column. A mixture of acetonitrile 0.2% (*v*/*v*) HCOOH in H_2O (gradient) was used as the elution phase. Measurements were carried out at $\lambda = 320$ and 280 nm. The identification of the compounds consisted of comparing the retention time of the tested peak with that of the standard (Sigma-Aldrich, Poznań, Poland). Only the bound fraction of phenolic acids was analyzed.

2.7. Determination of Flavonoids

Flavonoid composition was determined using the method described in Nowak et al. [29]. Extracted flavonoids were separated and identified using an Agilent UPLC equipped with a Nova-Pak C18 reversed-phase column (3.9 × 150 mm, particle size 5 µm; both from Waters, Milford, MA, USA). Solvent A was 0.3% (*v*/*v*) HCOOH in H_2O, while solvent B was HPLC-grade acetonitrile. The solvent flow rate was maintained at 1 mL/min. The gradient profile was as follows: 85% A at 0 min and 25% A at 40 min. The mobile phase of the gradient elution was as follows: A, acetonitrile with 0.1% formic acid; and B, 1% aqueous formic acid mixture (pH = 2). Chromatograms were recorded using a UV–Vis detector at $\lambda = 370$ nm. The separated compounds were identified by retention time mapping using a set of standards. The amount of the following flavonoids was determined using standard solutions (0.001–0.01 µg/mL) of the individual compounds (Sigma-Aldrich, Poznań, Poland). Seven flavonoids were determined: naringenin, vitaxin, rutin, quercetin, apigenin, kaempferol and lutein.

2.8. Statistical Analysis

Analysis of the results was performed using statistical inference methods [30,31]. The basic statistical descriptors included mean values and standard deviation (±SD). The normality of the distribution was tested with the W Shapiro–Wilk test, while the equality of variance in different samples was tested with a Levene test. Fixed factors were the year of study, species and nitrogen fertilization. For the studied features, multifactorial analysis of variance was used to find significant differences; in addition, the interaction of environmental conditions (year of study), genotype and nitrogen fertilization was determined for the quality of grains' features and environment and fertilization for physiological features for each genotype separately. Tukey's post hoc test was used to identify significant differences between means.

The similarities between the studied features and principal components were analyzed using an unweighted pair group method with the arithmetic mean (UPGMA) with Bray–Curtis (percent similarity) coefficients. Correspondence analysis (CA) was used to find the main gradients of the traits of the genotypes in the studied communities [32,33]. In the scatter plot, the 'joint plot' function was used to better visualize the distribution of the data. Canonical correspondence analysis (CCA) was used to visualize and identify factors correlating with study features depending on environmental conditions and nitrogen fertilization rate [33,34]. The Monte Carlo permutation test was used for testing the significance of canonical axes [31,35]. The level of significance for all statistical tests was accepted at $\alpha = 0.05$. The statistical calculations mentioned above were carried out with MS Excel 2019 software (Microsoft, Redmond, WA, USA, 2019), Statistica 13.3 (Dell, Round Rock, TX, USA, 2021), PAST 3.2 (Hammer UiO, 2018) and MVSP 3.2 (Multi Variate Statistical Package, Kovach Computing Services 2019) software.

3. Results

3.1. Physiological Parameters

3.1.1. *Hordeum vulgare* var. *rimpaui*

The weather conditions in the years of study had a significant impact on the SPAD leaf greenness index and PS II functioning indices (F_v/F_m and PI_{abs}) in the leaf development stage (BBCH 12) of *H. v.* var. *rimpaui*. In 2021, barley fertilized with nitrogen at rates of 30, 60 and 90 kg ha^{-1} was characterized by a significantly higher value of the SPAD index compared to the treatments fertilized and not fertilized with nitrogen in 2022. On the contrary, the values of the F_v/F_m index and PI_{abs} in 2022 (in all treatments) were higher than in 2021. The mineral nitrogen rate had no effect on the F_v/F_m index in any year of the study. In turn, the PI_{abs} index in 2022 with fertilization of 90 kg N ha^{-1} was significantly higher compared to the control (without mineral nitrogen fertilization) by 23% (Table 3).

In the shooting stage (BBCH 34), the values of SPAD indices in all treatments were higher in 2022 compared to 2021. In the first year of the study, the SPAD and F_v/F_m indices were significantly higher after the application of rates of 60 and 90 kg N ha^{-1} compared to the control. The F_v/F_m index was significantly higher at such fertilization rates, also compared to the rate of 30 kg N ha^{-1}. The PI_{abs} index in 2021 was significantly higher compared to the control by 41.7% only after applying a rate of 90 kg ha^{-1}. In 2022, fertilization with rates of 60 and 90 kg N ha^{-1} significantly increased the SPAD index and PI_{abs} compared to the control by 18.2% and 46.5% for the 60 kg ha^{-1} dose and 34.7% and 46.5% for the 90 kg ha^{-1} dose, respectively. Moreover, the SPAD value after the application of 90 kg N ha^{-1} was higher than after the application of 60 kg N ha^{-1}. In turn, the F_v/F_m index was significantly higher compared to the control after applying 30 and 90 kg N ha^{-1}. The F_v/F_m index was significantly higher in 2021 than in 2022 after the application of rates of 60 and 90 kg ha^{-1}. In turn, the PI_{abs} was significantly higher after applying 30, 60 and 90 kg N ha^{-1} in 2022 than in each treatment in 2021 (Table 3).

In the earing stage (BBCH57) in 2021, under the influence of fertilization with rates of 60 and 90 kg N ha^{-1}, the SPAD index significantly increased compared to the control. The PSII functioning indices did not respond significantly to nitrogen rates in 2021. In 2022, the SPAD value was significantly higher compared to the control under the influence of rates of 30, 60 and 90 kg ha^{-1} by 14.9%, 17.8% and 22.6%. In the analyzed year, nitrogen applied at a rate of 60 kg ha^{-1} significantly increased the PI_{abs} index compared to the control by 71.2%. The SPAD value was significantly higher in 2022 when fertilized with rates of 30, 60 and 90 kg ha^{-1} than in the entire range of rates (0–90 kg N ha^{-1}) in 2021. In turn, the F_v/F_m index was significantly higher under fertilization with rates of 60 and 90 kg N ha^{-1} in 2021 than in all treatments in 2022 (Table 3).

Table 3. Leaf greenness index (SPAD) and chlorophyll fluorescence parameters of *H. v.* var. *rimpaui*.

Stage	Year	Dose	SPAD	SD	F_v/F_m	SD	PI_{abs}	SD
BBCH 12	2021	0	373.7 ab	25.1	0.712 b	0.021	1.10 c	0.17
		30	451.7 a	47.6	0.701 b	0.025	1.02 c	0.20
		60	424.2 a	45.0	0.730 b	0.007	1.22 c	0.12
		90	446.0 a	62.8	0.710 b	0.031	1.19 c	0.30
	2022	0	265.3 c	26.7	0.798 a	0.004	3.17 b	0.24
		30	315.3 bc	30.3	0.799 a	0.004	3.54 ab	0.20
		60	300.3 bc	6.8	0.799 a	0.005	3.41 ab	0.13
		90	261.3 c	12.8	0.800 a	0.004	3.90 a	0.24
BBCH 34	2021	0	222.5 e	18.8	0.790 b	0.007	1.20 d	0.17
		30	251.8 de	34.5	0.789 b	0.004	1.47 cd	0.23
		60	299.0 d	36.3	0.803 a	0.002	1.89 cd	0.23
		90	311.2 d	28.1	0.808 a	0.010	2.06 c	0.33
	2022	0	386.0 d	28.1	0.772 c	0.006	2.15 bc	0.19
		30	436.0 bc	27.9	0.786 b	0.004	2.81 ab	0.34
		60	456.3 b	17.4	0.785 bc	0.012	3.15 a	0.67
		90	520.0 a	28.5	0.787 b	0.004	3.15 a	0.29
BBCH 57	2021	0	464.8 c	4.6	0.786 ab	0.005	2.98 b	0.47
		30	491.5 bc	25.3	0.795 ab	0.003	3.48 ab	0.59
		60	521.3 b	18.7	0.805 a	0.011	4.57 ab	0.88
		90	525.5 b	28.7	0.807 a	0.010	4.68 ab	0.82
	2022	0	504.7 bc	19.2	0.754 c	0.016	2.99 b	0.49
		30	579.7 a	10.2	0.771 bc	0.008	4.18 ab	0.32
		60	594.7 a	5.3	0.776 bc	0.017	5.12 a	1.11
		90	619.0 a	34.3	0.769 bc	0.002	4.43 ab	0.51

a–e mean values in column with common letters are not significantly different. MANOVA at the significance level $p = 0.05$; data are means; SD—standard deviation.

3.1.2. *Hordeum vulgare* var. *nigricans*

In the leaf development stage (BBCH 12) of *H. v.* var. *nigricans*, a significantly higher value of the SPAD index was recorded in the entire range of N rates analyzed in 2021 than in 2022. The nitrogen fertilization rate, regardless of the year, did not have a significant effect on this trait. The interaction of the year of study and nitrogen fertilization varied between the F_v/F_m and PI_{abs} indices. The F_v/F_m index in the control in 2022 was significantly higher than under fertilization with a rate of 60 kg N ha^{-1} in 2021. The PI_{abs} index under control conditions and with fertilization of 90 kg N ha^{-1} in 2022 was significantly higher than after applying 60 kg N ha^{-1} in 2021 (Table 4).

In the shooting stage (BBCH 34), both in 2021 and 2022, nitrogen fertilization at rates of 30, 60 and 90 kg ha^{-1} significantly increased the SPAD index compared to the control. In 2022, plants fertilized with rates of 30, 60 and 90 kg ha^{-1} were characterized by a significantly higher SPAD value than in 2021 in both those fertilized and not fertilized with nitrogen. A rate of 90 kg ha^{-1} in 2021 resulted in a significant increase in the value of the F_v/F_m index compared to the control. Moreover, the F_v/F_m index after applying the highest rate of nitrogen in 2021 was significantly higher than in each treatment in 2022. In the control group, in turn, the F_v/F_m index did not differ significantly between the years of the study. In the analyzed stage of plant development, nitrogen fertilization did not have a significant effect on the PI_{abs} index in 2021. In 2022, a significant increase of 28.9% in this parameter compared to the control was recorded under the influence of fertilization with rates of 90 kg N ha^{-1}. In 2022, after fertilization with 90 kg ha^{-1}, the PI_{abs} index was significantly higher than in 2021 in the rate range of 0–60 kg ha^{-1} (Table 4).

In the earing stage (BBCH 57), plants affected by nitrogen fertilization showed significant differences in the SPAD, F_v/F_m and PI_{abs} indices only in 2022. After fertilization with 90 kg N ha^{-1} in 2022, the plants were characterized by a significantly higher SPAD value than in the control by 27%. The plants did not respond by changes in the F_v/F_m to nitrogen fertilization in 2022, while the PI_{abs} significantly increased by 73.1% as affected by fertilization with a rate of 60 kg ha^{-1} compared to the treatment without nitrogen fertilization (Table 4).

Table 4. Leaf greenness index (SPAD) and chlorophyll fluorescence parameters of *H. v.* var. *nigricans*.

Stage	Year	Dose	SPAD	SD	F_v/F_m	SD	PI_{abs}	SD
BBCH 12	2021	0	497.0 a	45.6	0.742 ab	0.009	2.05 ab	0.33
		30	497.0 a	9.6	0.750 ab	0.001	2.19 ab	0.21
		60	484.5 a	13.3	0.728 b	0.034	1.80 b	0.33
		90	504.2 a	45.0	0.736 ab	0.006	1.86 ab	0.15
	2022	0	281.7 b	38.2	0.766 a	0.009	2.47 a	0.32
		30	257.7 b	35.5	0.759 ab	0.008	2.29 ab	0.25
		60	292.3 b	13.9	0.758 ab	0.009	2.24 ab	0.27
		90	245.0 b	38.3	0.763 ab	0.006	2.50 a	0.26
BBCH 34	2021	0	274.5 d	16	0.781 bc	0.004	1.58 c	0.17
		30	330.0 c	20.8	0.796 ab	0.005	2.33 bc	0.6
		60	353.5 bc	10.1	0.797 ab	0.009	2.21 c	0.66
		90	362 bc	17.3	0.803 a	0.002	2.48 abc	0.56
	2022	0	402.2 b	28.8	0.767 cd	0.008	2.15 c	0.49
		30	468.0 a	24.4	0.757 d	0.012	2.45 abc	0.27
		60	479.0 a	13.9	0.779 bc	0.015	3.69 ab	0.87
		90	518.7 a	36.4	0.781 bc	0.010	3.76 a	0.51
BBCH 57	2021	0	469.2 b	66	0.747 a	0.012	2.18 c	0.66
		30	523.5 ab	19.8	0.754 a	0.049	2.72 c	0.91
		60	530.2 ab	66.9	0.779 a	0.014	3.18 bc	0.91
		90	568.2 ab	26.9	0.765 a	0.019	3.15 bc	0.43
	2022	0	477.3 b	9.8	0.770 a	0.016	3.76 bc	0.61
		30	524.3 ab	41.1	0.788 a	0.003	5.04 ab	0.73
		60	553.7 ab	32.1	0.795 a	0.018	6.51 a	1.45
		90	606.3 a	21	0.790 a	0.012	4.80 ab	0.8

a–d mean values in column with common letters are not significantly different. MANOVA at the significance level $p = 0.05$; data are means; SD—standard deviation.

3.1.3. *Hordeum vulgare* var. *vulgare*

The interaction of the year of research and nitrogen fertilization had a significant impact on the SPAD, F_v/F_m and PI_{abs} indices of common barley (*H. v.* var. *vulgare*) in the leaf development stage (BBCH 12). There was no significant effect of nitrogen rates on the SPAD in BBCH 12 in the first year of the study. However, it is worth emphasizing that the SPAD index in the seedling stage was significantly higher in 2021 than in 2022. The F_v/F_m index in BBCH 12 in 2022 under the conditions of nitrogen application in the range of 0–90 kg ha^{-1} was significantly higher than in 2021 after the application of 30 kg N ha^{-1}. Moreover, in 2021, plants fertilized with a rate of 30 kg N ha^{-1} were also characterized by a significantly lower value of the SPAD index in relation to plants not fertilized with nitrogen and fertilized with 60 and 90 kg N ha^{-1}. The PI_{abs} index across the entire N rate range (0–90 kg ha^{-1}) was significantly higher in 2022 than in 2021. Fertilization with increasing rates of N did not affect the differentiation of the PI_{abs} index in the analyzed stage of plant development in 2022. In 2021, barley plants fertilized with a rate of 30 kg N ha^{-1} were characterized by a significantly lower PI_{abs} value compared with the treatment in which the rate was higher by 30 kg N ha^{-1} (Table 5).

In the shooting stage (BBCH 34), the SPAD value in 2022 in the rate range of 0–90 kg N ha^{-1} was significantly higher than in the control in 2021. Plants fertilized with rates of 60 and 90 kg N ha^{-1} in 2022 also showed a significantly higher SPAD compared to treatments fertilized in 2021 with rates of 30 and 60 kg ha^{-1}. The F_v/F_m index was mostly higher in 2021 than in 2022. Nitrogen fertilization did not significantly differentiate the PI_{abs} index in individual years of the study. Similarly, a significantly higher PI_{abs} value was recorded in 2022 compared to 2021 (Table 5).

Table 5. Leaf greenness index (SPAD) and chlorophyll fluorescence parameters of *H. v.* var. *vulgare*.

Stage	Year	Dose	SPAD	SD	Fv/Fm	SD	PI_{abs}	SD
BBCH 12	2021	0	512.0 a	17.8	0.754 ab	0.006	2.20 bc	0.17
		30	473.5 a	13.5	0.737 c	0.014	1.88 c	0.2
		60	489.5 a	14.6	0.758 ab	0.013	2.70 b	0.42
		90	482.0 a	21.4	0.748 ab	0.002	2.14 bc	0.18
	2022	0	352.7 bc	24.2	0.776 a	0.011	3.55 a	0.4
		30	383.7 b	43.1	0.778 a	0.016	3.45 a	0.31
		60	306.7 c	30.2	0.779 a	0.010	3.93 a	0.33
		90	341.0 bc	47.1	0.779 a	0.014	3.57 a	0.15
BBCH 34	2021	0	272.3 c	158.5	0.809 a	0.002	2.76 b	0.21
		30	353.0 bc	126.9	0.806 ab	0.006	2.63 b	0.34
		60	354.8 bc	109.7	0.812 a	0.006	2.79 b	0.25
		90	380.7 a–c	103.4	0.810 a	0.004	3.16 b	0.34
	2022	0	540.2 ab	25.5	0.790 bc	0.008	4.81 a	0.6
		30	537.8 ab	22.8	0.800 a–c	0.009	4.93 a	0.26
		60	606.5 a	34.1	0.785 c	0.01	4.69 a	0.71
		90	614.3 a	17.7	0.793 cb	0.006	5.48 a	0.58
BBCH 57	2021	0	470.0 c	76.4	0.778 a	0.006	2.22 b	0.5
		30	507.2 bc	54.2	0.809 a	0.012	4.08 ab	0.51
		60	535.5 a–c	25.7	0.774 a	0.042	3.68 ab	1.66
		90	562.5 a–c	39.6	0.815 a	0.014	5.26 a	1.36
	2022	0	497.3 bc	15.9	0.780 a	0.017	4.94 a	0.91
		30	534.7 a–c	53.9	0.764 a	0.024	3.95 ab	1.11
		60	598.3 ab	14.7	0.797 a	0.004	5.80 a	0.59
		90	619.3 a	29	0.795 a	0.010	5.64 a	0.46

a–c mean values in column with common letters are not significantly different. MANOVA at the significance level $p = 0.05$; data are means; SD—standard deviation.

In the earing stage (BBCH 57), in 2022, the SPAD value of plants fertilized with a rate of 90 kg ha^{-1} was significantly higher by 24.5% than in plants not fertilized with nitrogen. Nitrogen fertilization and the year of cultivation had no significant effect on the F_v/F_m. The interaction of these factors significantly diversified the PI_{abs} index, which only in 2021 was significantly higher in the treatment fertilized with a rate of 90 kg N ha^{-1} compared to the treatment without nitrogen fertilization (Table 5).

3.2. ABTS$^+$

H. v. var. *rimpaui* in 2021 was characterized by significantly higher ABTS$^+$ values in the absence of fertilization and when fertilizing with 90 kg ha^{-1} compared to doses of 30 and 60 kg ha^{-1}. In 2022, ABTS$^+$, regardless of the level of nitrogen fertilization, was significantly higher than in 2021. Moreover, in the second year of the study, nitrogen fertilization in the entire range of studied rates significantly increased the ABTS$^+$ of grain compared to the control. However, the highest antioxidant activity was observed in the treatments fertilized with a rate of 30 kg N ha^{-1}; it was significantly higher than after applying rates of 60 and 90 kg N ha^{-1}. In 2022, *H. v.* var. *rimpaui* unfertilized with nitrogen and after the application of the lowest rate of 30 kg N ha^{-1} showed significantly higher grain ABTS$^+$ values than the other genotypes (Table 6).

H. v. var. *nigricans* in 2021 responded to fertilization with a rate of 30 kg ha^{-1} with a significant increase in ABTS$^+$ compared to the control. However, increasing the N dose from 30 to 60 kg ha^{-1} and from 60 to 90 kg ha^{-1} resulted in a significant decrease in the ABTS$^+$ value. In 2022, fertilization with rates of 30, 60 and 90 kg ha^{-1} increased grain ABTS$^+$ compared to the control. Moreover, in the second year of the study, fertilization with 90 kg N ha^{-1} also significantly increased grain ABTS$^+$ compared to rates of 30 and 60 kg N ha^{-1}. *H. v.* var. *nigricans* was characterized by significantly higher grain ABTS$^+$ than other genotypes under fertilization conditions of 30 and 60 kg N ha^{-1} in 2021 and 90 kg N ha^{-1} in 2022 (Table 6).

Table 6. Antiradical capacity and total phenolic compounds in tested barley genotypes' grains.

Genotype	Year	Dose	ABTS (%)		SD	DPPH (%)		SD	TPC (mg GA/L)		SD
H. v. rimpaui	2021	0	15.5	n	0.1	41.3	b–g	27.3	39.7	l	0.9
		30	28.3	k	1.5	54.0	a–e	25.2	55.5	hi	2.2
		60	30.1	kj	0.8	59.0	a–d	27.2	52.1	i–k	2.6
		90	18.4	mn	0.4	71.7	ab	1.4	52.6	ij	1.4
	2022	0	47.5	f	0.4	27.8	e–h	0.6	52.2	i–k	0.6
		30	94.9	a	0.1	44.8	b–f	0.8	84.7	a	0.3
		60	63.7	d	0.7	34.0	d–h	0.6	66.3	de	0.4
		90	62.2	d	0.8	28.2	e–h	0.7	65.4	de	0.5
H. v. nigricans	2021	0	23.7	l	1.3	51.9	a–e	1.4	66.4	de	1.3
		30	51.9	e	1.4	50.5	a–e	1.4	70.5	c	1.2
		60	41.4	g	1.7	51.8	a–e	2.1	75.2	b	1.6
		90	30.2	k–i	0.7	46.8	a–e	1.2	81.2	a	1.7
	2022	0	4.9	o	0.4	6.5	h	0.4	35.1	mn	0.3
		30	33.8	ih	0.2	13.9	f–h	0.2	37.1	lm	0.2
		60	30.2	k–i	0.6	11.7	gh	0.0	34.1	mn	0.1
		90	78.8	b	0.7	29.5	e–h	0.6	68.1	cd	0.3
H. v. vulgare	2021	0	30.4	k–i	0.8	56.9	a–e	3.4	31.4	n	2.3
		30	40.9	g	1.9	63.5	a–c	4.1	60.0	fg	0.3
		60	33.3	j–h	0.6	75.1	a	0.9	40.5	l	1.0
		90	35.7	h	1.3	67.5	ab	1.9	63.1	ef	0.8
	2022	0	21.1	lm	0.3	12.6	gh	0.6	49.5	jk	0.8
		30	22.3	l	0.8	9.6	gh	0.6	48.4	k	0.1
		60	68.1	c	1.8	35.3	c–h	0.4	70.6	c	0.3
		90	29.4	k	1.4	13.5	gh	0.4	58.5	gh	0.6

a–o mean values in column with common letters are not significantly different. MANOVA at the significance level $p = 0.05$; data are means; SD—standard deviation.

In 2021, *H. v.* var. *vulgare* responded to fertilization with rates of 30 and 90 kg N ha^{-1} with a significant increase in ABTS$^+$ compared to the control. Increasing the rate from 30 to 60 kg ha^{-1} resulted in a significant reduction in the ABTS$^+$ of grain. In 2022, *H. v. vulgare* showed significantly lower ABTS$^+$ than in 2021 under fertilization conditions of 0, 30 and 90 kg ha^{-1}. The application of a rate of 60 kg N ha^{-1} in 2022 resulted in a significant increase in the antioxidant activity of grain compared to the control and a rate of 30 kg N ha^{-1}. Increasing the nitrogen rate from 60 to 90 kg ha^{-1} caused a decrease in the ABTS$^+$ of *H. v. vulgare* grain. This genotype, under conditions of no fertilization in 2021 and fertilization with a rate of 60 kg N ha^{-1} in 2022, was characterized by significantly higher ABTS$^+$ values than other barley genotypes (Table 6).

3.3. DPPH

The antioxidant activity of *H. v.* var. *rimpaui* grain expressed as DPPH varied by the interaction of the year of cultivation, genotype and nitrogen fertilization rates. At the application of the highest rate of 90 kg N ha^{-1}, the grain of *H. v.* var. *rimpaui* had significantly greater antioxidant activity in 2021 than in 2022. Only in the second year of the study was the DPPH value of the grain of *H. v.* var. *rimpaui* fertilized with 30 kg N ha^{-1} generally higher than in the other genotypes (Table 6).

H. v. var. *nigricans* did not show a significant response of DPPH antioxidant activity to nitrogen fertilization rates in 2021 or 2022. However, it was observed that this genotype was characterized by a higher grain DPPH value in 2021 compared to 2022 (Table 6).

H. v. vulgare also did not show significant differences in grain DPPH under the influence of increasing rates of nitrogen fertilization in both years of the study. However, it is worth noting that, as in *H. v.* var. *nigricans*, the antioxidant activity of DPPH in common barley was higher in 2021 than in 2022 (Table 6).

3.4. Total Phenolic Compounds

The interaction of the year of study, genotype and nitrogen fertilization significantly affected the total phenol content in grain. In both years of the study, the grain of *H. v.* var. *rimpaui* under the influence of fertilization with 30, 60 and 90 kg N ha^{-1} was characterized by a significantly higher total content of phenolic compounds compared to the control. In 2021, the nitrogen rate was not significant, while in 2022 the highest content of phenolic compounds was obtained after the application of 30 kg N ha^{-1}. Comparing the content of phenolic compounds in the grain of *H. v.* var. *rimpaui* between the years of study, with the same N rates, it can be concluded that 2022 was more favorable in this respect compared to 2021. In 2022, *H. v.* var. *rimpaui* fertilized with nitrogen usually contained more phenolic compounds than *H. v.* var. *nigricans* and *H. v.* var. *vulgare* (Table 6).

H. v. var. *nigricans* in 2021 responded to fertilization with a rate of 30 kg N ha^{-1} with a significant increase in the content of phenolic compounds in grain compared to the control. Increasing the dose from 30 to 60 kg ha^{-1} and from 60 to 90 kg ha^{-1} also significantly increased the content of these compounds. A higher content of phenolic compounds in the grain of *H. v.* var. *nigricans* was determined in 2021 compared to 2022 (except for the treatment with the application of 90 kg N ha^{-1}). Moreover, in the second year of the study, an increase in the content of phenolic compounds was noted after the application of a rate of 90 kg N ha^{-1} compared to the other treatments. In 2021, *H. v.* var. *nigricans*, regardless of the N fertilization rate, was characterized by a higher content of phenolic compounds in grain than the other studied genotypes. However, in 2022, comparing the same fertilization levels, *H. v.* var. *nigricans* showed a significantly lower content of these compounds than *H. v.* var. *rimpaui* and *H. v.* var. *vulgare* (Table 6).

In 2021, under the influence of nitrogen fertilization at rates from 30 to 90 kg ha^{-1}, *H. v. vulgare* accumulated more phenolic compounds in grain compared to the control. A similar relationship was observed in 2022, but only in relation to the application of higher nitrogen rates of 60 and 90 kg ha^{-1}. The comparison of treatments with the same N rate did not show any directional changes in the content of phenolic compounds in the compared years of study. *H. v. vulgare* in 2021 after the application of 30 and 90 kg N ha^{-1} contained significantly more phenolic compounds than *H. v.* var. *rimpaui.* In 2022, in turn, it was superior in this respect to *H. v.* var. *nigricans* cultivated at rates of 0, 30 and 60 kg N ha^{-1} (Table 6).

3.5. Total Protein

In no year of the study was the content of total protein in the grain of *H. v.* var. *rimpaui* differentiated by nitrogen fertilization. Only in 2022 did *H. v.* var. *rimpaui* fertilized with a rate of 90 kg N ha^{-1} contain significantly more protein compared to the control in 2021. In 2021, *H. v. rimpaui* fertilized with rates of 30 and 90 kg N ha^{-1} contained significantly more total protein than *H. v.* var. *nigricans* cultivated at 0, 30 and 90 kg N ha^{-1} and *H. v. vulgare* with no fertilization and fertilized with rates of 30 and 60 kg N ha^{-1} (Table 7).

The grain of *H. v.* var. *nigricans* in 2021 contained significantly more protein when fertilized with 60 kg N ha^{-1} compared to 30 kg ha^{-1}. Under fertilization with 0, 30 and 90 kg ha^{-1}, this genotype contained significantly more protein in 2022 compared to 2021. *H. v.* var. *nigricans* fertilized with a rate of 60 kg N ha^{-1} in 2021 usually contained more protein compared to *H. v. vulgare* (except for the treatment with the application of 90 kg N ha^{-1}) (Table 7).

H. v. vulgare in 2021, under the influence of a rate of 90 kg N ha^{-1}, significantly increased the content of phenolic compounds in grain compared to other treatments. In 2022, the protein content in grain in all treatments was similar. *H. v. vulgare* contained significantly more protein in grain in 2022 compared to 2021 in the dose range of 0, 30 and 60 kg ha^{-1}. This genotype fertilized with a rate of 90 kg N ha^{-1} in 2021 contained significantly more total protein in grain than *H. v.* var. *nigricans* fertilized with 30 kg N ha^{-1} (Table 7).

Table 7. Total flavonoid and phenolic acid content in tested barley genotypes' grains.

Genotype	Year	Dose	Flavonoids	SD	Phenolic Acids	SD	Total Protein	SD
H. v. rimpaui	2021	0	531.9 b	13.5	1446.7 e	9.5	97.9 b–e	0.5
		30	542.6 b	30.3	1461.6 e	57.3	113.5 ab	19.4
		60	538.5 b	18.1	1437.9 e	20.3	106.4 a–d	14.7
		90	534.2 b	8.5	1485.6 c–e	27.5	120.0 ab	4.0
	2022	0	540.6 b	40.1	1524.4 b–e	191.3	114.5 ab	1.9
		30	530.0 b	20.9	1470.6 de	55.8	110.6 a–c	1.5
		60	506.6 b	21.7	1440.9 e	21.8	116.8 ab	0.3
		90	525.9 b	39.2	1551.1 b–e	75.2	127.3 a	1.4
H. v. nigricans	2021	0	598.1 ab	21.2	1778.1 a	32.3	86.7 d–f	1.9
		30	575.4 b	27.1	1779.1 a	32.9	66.2 f	7.8
		60	546.7 b	31.1	1789.5 a	70,0	107.8 a–d	6.9
		90	598.4 ab	30.7	1836.9 a	46.9	89.2 c–f	24.0
	2022	0	585.9 ab	24.9	1675.1 a–c	132.4	107.6 a–d	3.8
		30	596.6 ab	20.0	1753.1 a	39.2	106.6 a–d	1.4
		60	681.2 a	159.9	1660.2 a–d	113.1	119.4 ab	1.9
		90	585.7 ab	27.8	1708.8 ab	144.9	122.2 a	0.6
H. v. vulgare	2021	0	147.3 c	2.5	757.3 f	35.9	77.5 ef	3.0
		30	143.1 c	4.2	753.2 f	37.0	81.6 ef	10.6
		60	146.9 c	4.6	764.3 f	24.3	81.4 ef	2.1
		90	132.9 c	6.9	783.8 f	14.1	106.9 a–d	11.1
	2022	0	145.0 c	2.6	728.1 f	21.3	105.5 a–d	4.1
		30	142.6 c	5.3	678.5 f	45.0	106.4 a–d	5.0
		60	141.8 c	3.8	636.3 f	14.2	119.1 ab	6.3
		90	138.8 c	4.8	702.5 f	51.7	122.5 a	2.4

a–f mean values in column with common letters are not significantly different. MANOVA at the significance level $p = 0.05$; data are means; SD—standard deviation.

3.6. Total Flavonoids and Phenolic Acids

Nitrogen fertilization and the year of cultivation did not significantly differentiate the content of phenolic acids and flavonoids in the grain of *H. v. rimpaui*. This genotype, regardless of fertilization and the year of cultivation, contained significantly more phenolic acids and flavonoids than *H. v. vulgare* (Table 7).

Similarly, *H. v. nigricans* in most cases did not respond to fertilization and the year of cultivation with differences in the content of phenolic acids and flavonoids in the grain. Only when fertilized with a rate of 60 kg ha^{-1}, it accumulated significantly more flavonoids in the grain in 2022 than in 2021. *H. v.* var. *nigricans*, regardless of nitrogen fertilization and the year of study, contained significantly more flavonoids and phenolic acids in the grain than *H. v. vulgare*. In 2021, regardless of the level of nitrogen nutrition, *H. v.* var. *nigricans* also contained significantly more phenolic acids than *H. v. rimpaui*. In 2022, a similar relationship between genotypes was observed when fertilizing with rates of 30 and 60 kg N ha^{-1}.

The content of phenolic acids and flavonoids in the grain of *H. v. vulgare* was the lowest among the studied genotypes and was not significantly differentiated by nitrogen fertilization or the year of cultivation (Table 7).

3.7. Correlations

Correlation analysis of physiological and qualitative characteristics of *H. v. rimpaui* showed a positive relationship between ABTS^{+} as well as TPC and the SPAD value in each of the analyzed development stages (BBCH 34 and BBCH 57). Moreover, the above-mentioned grain quality characteristics were positively correlated with the PI$_{abs}$ in BBCH 34. In contrast, a negative relationship between DPPH and the SPAD value as well as the PI$_{abs}$ was demonstrated in the stage under discussion. In both development stages, the DPPH parameter was positively and ABTS^{+} negatively correlated with the F_V/F_M index.

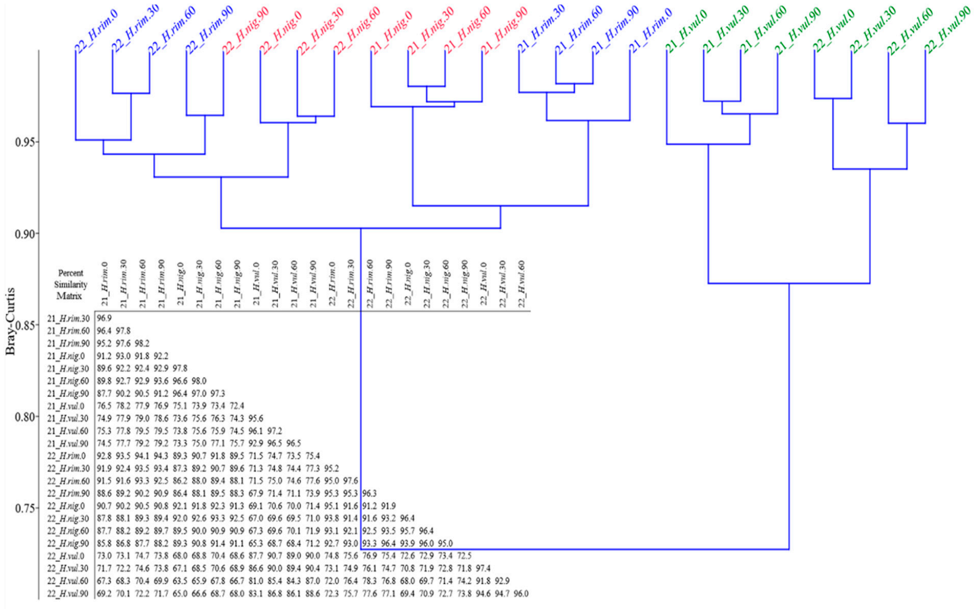
Analysis of the relationship between plant parameters and the grain of *H. v. nigricans* showed, similarly to *H. v. rimpaui*, a positive correlation of ABTS^{+} with the SPAD value in both developmental stages and with the PI_{abs} in BBCH 34. Similarly to the previously described genotype, DPPH was negatively correlated with the SPAD and with the PI_{abs} in BBCH 34. In this development stage, a positive relationship between DPPH and TPC and the F_v/F_M index was also demonstrated. Among the compared genotypes, only in *H. v.* var. *nigricans* was a positive correlation of the flavonoid content with the PI_{abs} index observed in BBCH 34 as well as in BBCH 57.

The analysis of the relationship between the physiological and qualitative characteristics of *H. v. vulgare* grain indicates, similarly to *H. v.* var. *nigricans,* the negative correlation of DPPH with the SPAD value in BBCH 34 and with the F_v/F_M and PI_{abs} in BBCH 57. A positive correlation of DPPH with the F_v/F_M, similarly to *H. v.* var. *nigricans*, was shown in BBCH 34. TPC in *H. v. vulgare* was positively correlated with the PI_{abs} in BBCH 34 and with the SPAD value in BBCH 57.

In all studied barley genotypes, the content of total protein in the grain was positively correlated with the SPAD value in BBCH 34 and with the PI_{abs} index in the BBCH 34 and BBCH 57 stages.

3.8. Cluster and Correspondence Analysis

Quantitative similarity analysis based on Bray–Curtis distances divided the data according to variability into two large groups, the first of which was based on the primary genotypes of *H. v. nigricans* and *H. v. rimpaui*; the second one was composed of *H. v. vulgare*. These groups consisted of five smaller subgroups, the division of which was affected by the year of study (Figure 1). The similarity depended the least on the rate of nitrogen fertilization.



Percent Similarity Matrix	21_H.rim.0	21_H.rim.30	21_H.rim.60	21_H.rim.90	21_H.nig.0	21_H.nig.30	21_H.nig.60	21_H.nig.90	21_H.vul.0	21_H.vul.30	21_H.vul.60	21_H.vul.90	22_H.rim.0	22_H.rim.30	22_H.rim.60	22_H.rim.90	22_H.nig.0	22_H.nig.30	22_H.nig.60	22_H.nig.90	22_H.vul.0	22_H.vul.30	22_H.vul.60
21_H.rim.30	96.9																						
21_H.rim.60	96.4	97.8																					
21_H.rim.90	95.2	97.6	98.2																				
21_H.nig.0	91.2	93.0	91.8	92.2																			
21_H.nig.30	89.6	92.2	92.4	92.9	97.8																		
21_H.nig.60	89.8	92.7	92.9	93.6	96.6	98.0																	
21_H.nig.90	87.7	90.2	90.5	91.2	96.4	97.0	97.3																
21_H.vul.0	76.5	78.2	77.9	76.9	75.1	73.9	73.4	72.4															
21_H.vul.30	74.9	77.9	79.0	78.6	73.6	75.6	76.3	74.3	95.6														
21_H.vul.60	75.3	77.8	79.5	79.5	73.8	75.6	75.9	74.5	96.1	97.2													
21_H.vul.90	74.5	77.7	79.2	79.2	73.3	75.0	77.1	75.7	92.9	96.5	96.5												
22_H.rim.0	92.8	93.5	94.1	94.3	89.3	90.7	91.8	89.5	71.5	74.7	73.5	75.4											
22_H.rim.30	91.9	92.4	93.5	93.4	87.3	89.2	90.7	89.6	71.3	74.8	74.4	77.3	95.2										
22_H.rim.60	91.5	91.6	93.3	92.5	86.2	88.0	89.4	88.1	71.5	75.0	74.6	77.6	95.0	97.6									
22_H.rim.90	88.6	89.2	90.2	90.9	86.4	88.1	89.5	88.3	67.9	71.4	71.1	73.9	95.3	95.3	96.3								
22_H.nig.0	90.7	90.2	90.5	90.8	92.1	91.8	92.3	91.3	69.1	70.6	70.0	71.4	95.1	91.6	91.2	91.9							
22_H.nig.30	87.8	88.1	89.3	89.4	92.0	92.6	93.3	92.5	67.0	69.6	69.5	71.0	93.8	91.4	91.6	93.2	96.4						
22_H.nig.60	87.7	88.2	89.2	89.7	89.5	90.0	90.9	90.9	67.3	69.6	70.1	71.9	93.1	92.1	92.5	93.5	95.7	96.4					
22_H.nig.90	85.8	86.8	87.7	88.2	89.3	90.8	91.4	91.1	65.3	68.7	68.4	71.2	92.7	93.0	93.3	96.4	93.9	96.0	95.0				
22_H.vul.0	73.0	73.1	74.7	73.8	68.0	68.8	70.4	68.6	87.7	90.7	89.0	90.0	74.8	75.6	76.9	75.4	72.6	72.9	73.4	72.5			
22_H.vul.30	71.7	72.2	74.6	73.8	67.1	68.5	70.6	68.9	86.6	90.0	89.4	90.4	73.1	74.9	76.1	74.7	70.8	71.9	72.8	71.8	97.4		
22_H.vul.60	67.3	68.3	70.4	69.9	63.5	65.9	67.8	66.7	81.0	85.4	84.3	87.0	72.0	76.4	78.3	76.8	68.0	69.7	71.4	74.2	91.8	92.9	
22_H.vul.90	69.2	70.1	72.2	71.7	65.0	66.6	68.7	68.0	83.1	86.8	86.1	88.6	72.3	75.7	77.6	77.1	69.4	70.9	72.7	73.8	94.6	94.7	96.0

Figure 1. Results of cluster analysis based on Bray–Curtis distance. Red—*H. v. nigricans*; Blue—*H. v. rimpaui*; Green—*H. v. vulgare*.

Correspondence analysis (CA) explained a total of 91.9% of the total variation (Figure 2). The analysis indicated different reactions in terms of the total content of phenolic acids and flavonoids compared to the other tested traits in the context of the analyzed factors (the year of study, cultivar and rates of nitrogen fertilization). It is clearly visible that the content of phenolic acids and flavonoids was more related to *H. v. rimpaui* and *H. v. nigricans* than to *H. v. vulgare.*

The distribution of mutual relations strongly differentiated the SPAD index in BBCH 12 and 34 and the PI_{abs} from all three measurement dates. Most of the studied physiological and qualitative characteristics of grain (except for the content of flavonoids and phenolic acids) were more related to *H. v. vulgare*, regardless of the amount of nitrogen fertilization applied.

The analysis also confirmed clear differences between the years of study, especially for *H. v. vulgare*. The CA also shows that most of the examined traits are differentiated to a lesser extent in *H. v.* var. *nigricans* and *H. v. rimpaui*.

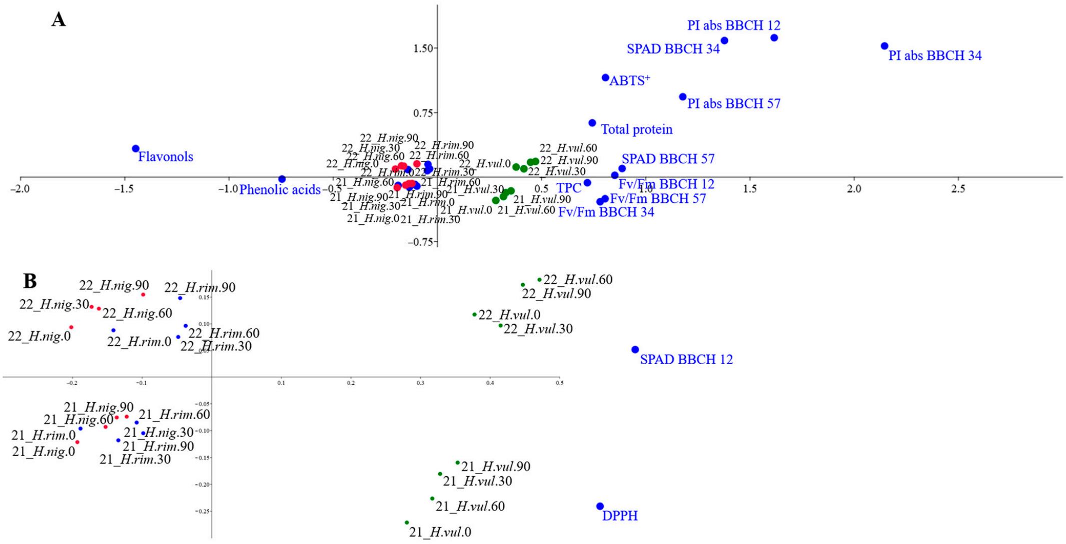
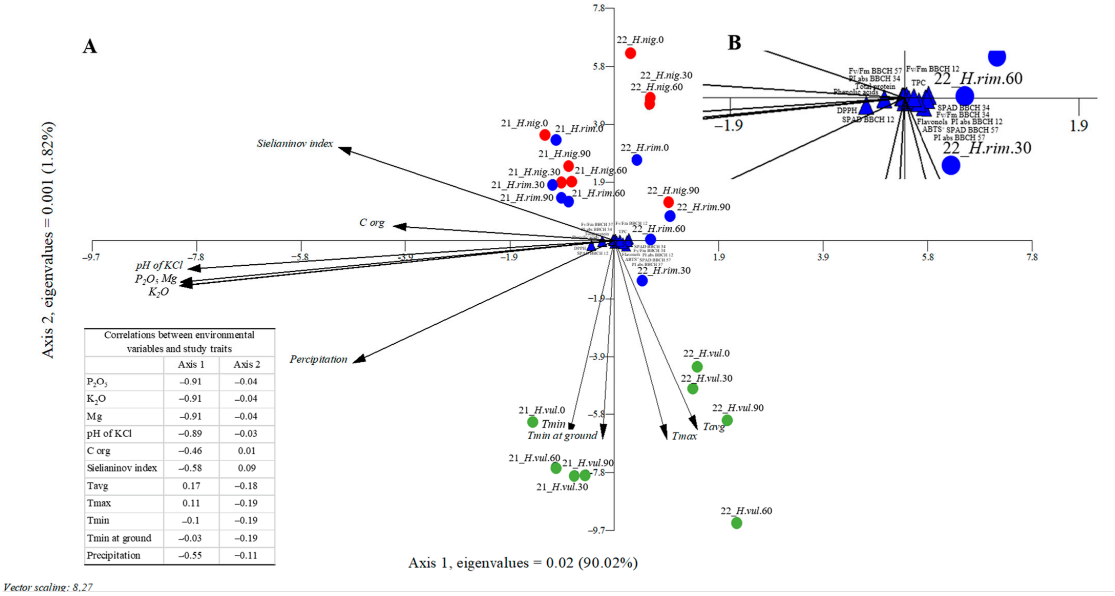


Figure 2. (**A**) Results of the correspondence analysis; (**B**) enlarged central part of (**A**).

3.9. Canonical Analysis

The results of canonical analysis (CCA) indicate that environmental factors differentiated in the years of study clearly group together and have a stronger impact on the tested genotypes than the nitrogen rate. *H. v. vulgare* was most affected by temperature during the growing season. The analysis indicates that this factor had an even, similar effect on all assessed physiological and qualitative characteristics of *H. v. vulgare*.

Primary genotypes, especially *H. v. rimpaui* fertilized with rates of 30, 60 and 90 kg N ha^{-1}, responded more strongly to soil factors, especially pH and the content of magnesium, phosphorus and potassium, and to a lesser extent organic carbon (Figure 3).



Correlations between environmental variables and study traits		
	Axis 1	Axis 2
P_2O_5	−0.91	−0.04
K_2O	−0.91	−0.04
Mg	−0.91	−0.04
pH of KCl	−0.89	−0.03
C org	−0.46	0.01
Sielianinov index	−0.58	0.09
Tavg	0.17	−0.18
Tmax	0.11	−0.19
Tmin	−0.1	−0.19
Tmin at ground	−0.03	−0.19
Precipitation	−0.55	−0.11

Figure 3. (**A**) Results of the canonical analysis (significance of first axis: Monte Carlo permutation test (F-ratio = 3.31, p = 0.048); significance of second axis: Monte Carlo permutation test (F-ratio = 1.01, p = 0.154)); (**B**) enlarged central part of (**A**).

The total precipitation and the Sielaninov coefficient were slightly less important, with the total precipitation having a stronger impact on *H. v. vulgare*. The above results are also reflected in the correlation of the effects of environmental factors with the analyzed main components, and thus also with the studied traits.

4. Discussion

Nitrogen is one of the main factors regulating the functioning of the photosynthetic apparatus, which, among other things, increases the rubisco content in the leaves and the quantum yield of the plant [36]. The results obtained in our study confirm the effect of nitrogen fertilization on the chlorophyll content in leaves and the efficiency of photosynthesis. This factor had the greatest impact on the SPAD and PI_{abs} indices of the studied genotypes and, to a slightly lesser extent, on the F_v/F_m. Similarly, Lin et al. [37] studying oats and Tanray et al. [36] in studies on rice proved that nitrogen fertilization had a stimulating effect on all PSII functioning indices, including quantum efficiency and the F_v/F_m.

However, the physiological response of barley to nitrogen fertilization observed in our study depended on the study year, the genotype and its development stage. As a rule, no significant effect of nitrogen fertilization on the physiological indices of barley seedlings was observed in individual years of the study. *H. v.* var *rimpaui* fertilized with nitrogen was characterized by a significantly higher SPAD index than in the control without nitrogen only in the shooting and earing stages and PI_{abs} in BBCH 34 in both years of the study. *H. v.* var *nigricans* showed a similar reaction only in 2022, which was characterized by lower rainfall during the growing season. The increase in the above-mentioned indices depended on the nitrogen rate and was most often observed when fertilizing with 60 and 90 kg N ha^{-1}. In 2022, both genotypes (*H. v.* var. *rimpaui* and *H. v.* var. *nigricans*) under the influence of fertilization with 60 kg N ha^{-1} also showed a higher PI_{abs} in the earing stage compared to the control without nitrogen. The nitrogen fertilization of the discussed genotypes, in particular with a rate of 90 kg N ha^{-1}, increased the F_v/F_m value in the shooting stage compared to the control. The stimulating effect of mineral nitrogen fertilization on the efficiency of photosynthesis and the chlorophyll content in cereal leaves is also confirmed by Kubar et al. [38] and Noor et al. [39]. Nitrogen fertilization is a stimulant that accumulates in plants for the synthesis of pigments such as Chl a, Chl b, total chlorophyll and carotenoids and increases the stomatal conductance, photosynthetic rate, intercellular CO_2 and transpiration rate, resulting in more efficient photosynthesis [38]. Similar results were also obtained by Lin et al. [37], who, when fertilizing oats with rates in the range of 60–120 kg N ha^{-1}, observed an increase in photosynthetic parameters and chlorophyll content in leaves and indicated 90 kg N ha^{-1} as the most optimal rate.

H. v. vulgare responded to nitrogen fertilization with much less variation in the potential chlorophyll content in leaves (SPAD) and PSII functioning indices. In the earing stage, only when fertilizing with 90 kg N ha^{-1} did the PI_{abs} index increase significantly in the first year and the SPAD index in the second year of the study compared to the control. Similarly, in terms of the F_v/F_m, no significant differentiation was observed under nitrogen fertilization in 2021 and 2022. Fradgley et al. [40], using wheat as an example, proved that breeding processes have resulted in modern, intensive cultivars having a much wider root system than older ones, which may result in the increased uptake of nutrients from the soil. Therefore, in our study it is possible *H. v. vulgare* was able to take up and manage soil nitrogen to a greater extent in conditions of no fertilization or low rates of this nutrient, which resulted in it maintaining a relatively high efficiency of photosynthesis in such conditions and having no significant response to fertilization with moderate rates of nitrogen. According to Szczepanek et al. [23], *H. v.* var. *rimpaui* creates a large plant biomass in conditions of optimal humidity. The optimal humidity conditions observed in 2021 in our study could therefore have contributed to an increase in plant biomass, which translated into reduced nitrogen concentration in leaves and lower SPAD and PI_{abs} indices compared to 2022. This thesis may be confirmed by the fact that fertilization with a rate of 90 kg N ha^{-1}

increased the SPAD and PI_{abs} to the level achieved by plants in 2022 in control treatments, and sometimes by those fertilized with a rate of 30 kg N ha^{-1}. Shangguan et al. [41], examining the effect of nitrogen fertilization on wheat under conditions of full irrigation and water deficit, found that water deficiency strongly reduced the chlorophyll content in leaves and the efficiency of photosynthesis, compared to conditions of full water availability; however, nitrogen fertilization increased the photosynthetic functioning indices.

In our study, the leaf greenness index and the F_v/F_m were also shaped by the environmental conditions observed in individual years of the study. In 2021, *H. v. rimpaui* plants regardless of fertilization, as well as *H. v.* var. *nigricans* and *H. v. vulgare* fertilized with moderate rates of nitrogen, were characterized by a significantly lower Fv/Fm, and at the same time a higher SPAD in the seedling stage than in 2022. The higher concentration of chlorophyll in the leaves in the first year of the study could result from optimal humidity conditions and the greater availability of nitrogen from the soil. This year, however, severe frosts were observed during the emergence period, which could have resulted in damage to photosystem II and a reduction in the maximum quantum efficiency of photosynthesis (F_v/F_m), especially in nitrogen-fertilized treatments. In 2021, plants of all tested genotypes were characterized by a higher F_v/F_m in the shoot stage than in 2022, which could be due to much higher rainfall and thus a more favorable hydrothermal index for plants in this period than in 2022. Maximum photosynthetic efficiency (F_v/F_m) is a very sensitive index of the chemical activity of the photosynthetic apparatus. In most plant species in the full development stage and growing in optimal environmental conditions, this parameter has a value of 0.83. A decrease in the value of this index indicates the impact of stress factors that have damaged the PSII function, which is particularly visible in plants growing under conditions of simultaneous exposure to drought and high light intensity [23,41]. In our study, such conditions were observed in 2022, which is confirmed by the low Sielianinov coefficient (Table 1).

In 2022, SPAD and PI_{abs} values were generally significantly higher in the generative development stages than in 2021 in all studied genotypes. However, nitrogen fertilization at rates of 60 and 90 kg N ha^{-1} in the first year of the study generally resulted in an increase in the SPAD and PI_{abs} to values similar to those determined in the second year in control treatments or those fertilized with a rate of 30 kg N ha^{-1}. This effect was much more pronounced in *H. v.* var. *rimpaui* than in *H. v.* var. *nigricans* and *H. v. vulgare*, which was characterized by the lowest variability of the SPAD and PI_{abs} between years. Differences in the response of barley plants to environmental conditions that varied over the years of the study and varied nitrogen supply could result from different morphological and physiological characteristics between the studied genotypes. The primary barley genotypes are characterized by a higher production of plant biomass than modern genotypes, although they are characterized by the greater stability of the functioning of photosystem II in unfavorable hydrothermal conditions, but a larger plant biomass increases the demand for nutrients, which translates into a lower concentration of chlorophyll in the leaves [23].

Nitrogen fertilization significantly increased the content of total phenolic compounds and the $ABTS^+$ antioxidant potential of the grains of the studied genotypes. However, the response of individual genotypes differed depending on the rate and year of cultivation. *H. v. rimpaui* in both years of the study, under the influence of nitrogen fertilization, increased the content of total phenolic compounds in grain and the $ABTS^+$ antioxidant potential compared to the control. The highest concentration of these compounds in the grain of *H. v. rimpaui* was obtained when fertilizing with rates of 30 and 60 kg N ha^{-1} in 2021 and 30 kg N ha^{-1} in 2022. *H. v.* var. *nigricans* in 2021, with an increase in the rate, increased the accumulation of phenolic compounds in grain, reaching their highest concentration when fertilized with 90 kg N ha^{-1}. The beneficial effect of a rate of 90 kg N ha^{-1} on TPC in the grain of this genotype was also confirmed in 2022. The grain of *H. v.* var. *nigricans*, however, showed the highest $ABTS^+$ antioxidant potential when fertilized with 30 kg N ha^{-1} in the first and 90 kg N ha^{-1} in the second year of the study. *H. v. vulgare* was similarly characterized by the highest concentration of total phenolic compounds in grain under fertilization conditions of 30 and 90 kg N ha^{-1} in 2021, characterized by optimal hydrothermal conditions. In dry 2022, an increase in this parameter compared to the control was achieved after the application of 60 and 90 kg N ha^{-1}, with the maximum being achieved when fertilizing with 60 kg N ha^{-1}. Nitrogen availability affects the activity of L-phenylalanine ammonia lyase (PAL), the enzyme responsible for catalyzing the ammonia elimination reaction from aromatic amino acids (phenylalanine and tyrosine) in the phenylpropanoid pathway, which in turn leads to the formation of cinnamic acid, a substrate in the biosynthesis of many phenolic compounds [16]. Ma et al. [42] report that both nitrogen fertilization and optimal water supply to plants resulted in an increase in the content of the free fractions of phenolic acids in wheat grain and its antioxidant potential in relation to treatments without N fertilization and treatments grown under drought conditions. The above-mentioned authors, despite the significant interaction between both factors, failed to establish a clear direction of the changes in the chemical composition of grain resulting from it.

In our study, the DPPH antioxidant potential of the grains of the studied genotypes was not significantly differentiated by nitrogen fertilization in individual study years, as well as the content of the fraction of bound phenolic acids and flavonoids. The grain of the studied genotypes was characterized by a significantly higher DPPH antioxidant potential in 2021 than in 2022. *H. v. rimpaui* and *H. v. nigricans* under fertilization conditions with a rate of 30 kg N ha^{-1} were characterized by significantly higher $ABTS^+$ and DPPH antioxidant potential than *H. v. vulgare*. Primary genotypes also generally contained significantly more phenolic acids and flavonoids than the cultivated variety. The most total phenolic compounds in 2021 were determined in the grain of *H. v.* var. *nigricans*. Interestingly, in the second year of the study, this genotype was characterized by the lowest concentration of these compounds among the studied genotypes. In the second year of the study, *H. v. rimpaui* contained significantly more TPC than the other genotypes.

The concentration of phenolic acids and flavonoids was differentiated mainly by genotype and depended to a lesser extent on agrotechnical and environmental factors. According to Stumpf et al. [18], fertilization with increasing rates of nitrogen increased the content of phenolic compounds in immature wheat grain, but only the concentration of the free fractions was changed, while the insoluble fraction did not show any response to this factor. In our study, only the bound fractions of phenolic acids and flavonoids were determined using the HPLC method. This means that, similarly to the study by Stumpf et al. [18], the change in TPC content should probably be related only to the free fraction of phenolic compounds. In a study by Kwiatkowski et al. [43], they also failed to confirm the effect of agrotechnical factors on the removal potential of barley grain DPPH.

H. v. rimpaui and *H. v.* var. *nigricans* did not show a significant response to nitrogen fertilization in terms of protein content in grain in individual years of cultivation. However, it significantly increased the total protein content in grain compared to the control without fertilizer only in *H. v. vulgare* in 2021 after the application of 90 kg N ha^{-1}. *H. v. rimpaui* fertilized with a rate of 30 kg N ha^{-1} and *H. v.* var. *nigricans* when fertilizing with rates in the range of 0–60 kg N ha^{-1} contained significantly more protein than *H. v. vulgare* grown at 0, 30 and 60 kg N ha^{-1}. The use of the highest studied nitrogen rate in 2021 resulted in a significantly higher protein content in the grain of *H. v. vulgare* compared to *H. v. nigricans*. The positive effect of nitrogen fertilization on the protein content in cereal grains has been confirmed many times in other studies, including Boulelouach et al. [44]. It should be noted, however, that among the studied genotypes, only *H. v. vulgare* responded with a significant increase in the protein content in the grain, and this was affected by the highest studied rate; the remaining treatments, especially the primary genotypes, showed only a non-significant increasing tendency in this respect. Perhaps this indicates a greater ability of the modern cultivar *H. v. vulgare* to use nitrogen fertilization, especially large rates, and convert it into protein compared to the primary genotypes. This assumption has its justification, among others: in the study by Cormier et al. [45], they report that modern cultivars are usually selected under conditions of high N supply; therefore, they do not always have to perform well under low N conditions, but they respond well to high fertilization. According to the authors, the G x N interaction (genotype x nature) also has an impact on the agronomic traits of plants. In our study, the grain of the studied genotypes was generally characterized by a significantly higher protein content in the grain in 2022 than in 2021, which could be related to weather conditions in the study years and generally higher SPAD and PI_{abs} indices in the second year of the study.

The content of total phenolic compounds and the $ABTS^+$ antioxidant potential of *H. v. rimpaui* grain were positively correlated with the SPAD and PI_{abs} in the shooting and earing stages. Similarly, *H. v.* var. *nigricans* was characterized by a positive relationship between $ABTS^+$ and the SPAD in the generative development stages as well as the PI_{abs} in the shooting stage. In this genotype, TPC was positively correlated with the F_v/F_m in the shooting stage. *H. v. vulgare* was characterized by a positive correlation between TPC with the PI_{abs} in BBCH 34 and the SPAD in BBCH 57. The DPPH antioxidant potential was negatively associated with the SPAD and PI_{abs} in the generative developmental stages in all studied genotypes. *H. v.* var. *rimpaui* and *H. v.* var. *nigricans* were also characterized by a negative correlation of this parameter with the F_v/F_m in the generative period of plant growth. By contrast, *H. v. vulgare* showed a positive correlation between these traits. Protein content also significantly and positively depended on the SPAD and PI_{abs} in the generative development stages of *H. v.* var. *rimpaui* plants.

As described earlier, nitrogen fertilization had a positive effect on the SPAD leaf greenness index, and thus the PI_{abs} functioning index, as well as grain quality traits such as protein, TPC and $ABTS^+$ antioxidant potential. The availability of nitrogen is necessary for proper metabolism and affects the higher level of chlorophyll and rubisco in the leaves, the efficiency of photosynthesis and the transport of carbon and nitrogen to the seeds, so it stimulates the protein content in the seeds [46]. Similarly to proteins, phenolic compounds, constituting the largest group of secondary metabolites, are synthesized from aromatic amino acids, and therefore from nitrogen. However, this process takes place in the phenylpropanoid pathway with the participation of the enzyme phenylalanine ammonia lyase, the activity of which is blocked by an increased supply of nitrogen [21]. According to these authors, nitrogen fertilization limits the concentration of phenolic compounds in plants in a rate-dependent manner. In a study by Zrckova et al. [47], significantly more phenolic compounds were observed in grain in a very dry crop year than in a year with optimal rainfall. In our study, in the second year of cultivation characterized by an unfavorable hydrothermal coefficient, a significantly lower F_v/F_m index was observed throughout most of the growing period. Low values of the F_v/F_m index indicate the ongoing photoinhibition process (the inhibition of photosynthesis and damage to the photosynthetic apparatus at high light intensities). If, in drought conditions, the power antennas receive too much energy, there may be an overproduction of the triplet states of chlorophyll, which promotes the production of singlet oxygen (O_2), which is a highly reactive form of oxygen [48–50]. Such conditions may stimulate the synthesis of phenolic compounds by plants, which in this study resulted in their higher concentration in the grain and increased antioxidant potential. Thus, the relationship between the assessed physiological parameters SPAD, PI_{abs} and F_v/F_m, shaped by the interaction of environmental factors, nitrogen fertilization and genotype, is reflected in the chemical composition and antioxidant activity of barley grain.

This is also confirmed by the results of multivariate analyses, which indicate that the SPAD and PI_{abs} indices had the greatest impact on the distribution of mutual relations. The analysis also confirms the significant importance of the F_v/F_m in shaping total phenolic compounds in the grain of the studied genotypes. Analyses indicate that of the greatest importance in shaping the physiological and qualitative characteristics of grain are genetic factors and their response to environmental factors, and agrotechnical factors such as the rate of nitrogen fertilization are less important. *H. v. vulgare* was more susceptible to environmental factors than the primary genotypes, especially to temperature and rainfall. *H. v. rimpaui* and *H. v. nigricans* were more influenced by soil conditions, and above all by pH and the content of Mg, P and K.

To sum up, the primary factor shaping the chemical composition of grain is genetic traits. However, the studied genotypes responded in different ways to environmental and agrotechnical factors. Nitrogen fertilization had a stimulating effect on the leaf greenness index and PSII functioning indices, which translated into an increased content of phenolic compounds and protein in the barley grain. The accumulation of TPC and protein in the grain is probably related or occurs simultaneously and responds positively to nitrogen fertilization because it improves the leaf greenness index (SPAD) and the PSII functioning index (PI_{abs}). However, the response of individual genotypes to the rate of nitrogen fertilization and the storage of TPC and protein in their grains is strongly related to environmental factors. The increase in the PI_{abs} associated with a higher SPAD results in the increased antioxidant activity of the grain, especially in unfavorable environmental conditions in which the maximum efficiency of photosynthesis (F_v/F_m) is limited, which may indicate the ongoing process of photoinhibition and the formation of reactive oxygen species, which in turn stimulates the plant for the biosynthesis of phenolic compounds and increases their concentration in the grain and increases the antioxidant potential.

5. Conclusions

The content of phenolic compounds and protein in the grain and its antioxidant potential depended most on the genotype, then on the environment, and least on nitrogen fertilization. However, the use of nitrogen fertilizer significantly increased the leaf greenness index (SPAD), the PSII functioning index (PI_{abs}) and the PSII maximum quantum efficiency (F_v/F_m) and increased the $ABTS^+$ antioxidant potential of grain as well as the total content of phenolic compounds in the grain. The protein content increased under fertilization with the highest studied rate of 90 kg N ha^{-1} only in *H. v. vulgare*. The response to N fertilization depended on its interaction with environmental conditions and genotype. Unfavorable weather conditions observed in the second year of the study resulted in a decrease in the F_v/F_m and an increase in the SPAD and PI_{abs}. Under these conditions, higher $ABTS^+$ antioxidant activity and total protein content in the grain were also observed. The primary genotypes were characterized by significantly higher $ABTS^+$ antioxidant activity and content of phenolic compounds in the grain, and they responded more strongly to nitrogen fertilization than the modern cultivar. *H. v. vulgare* was characterized by smaller differences in the SPAD, PI_{abs} and F_v/F_m index than the primary genotypes with variable nitrogen availability, but it was more sensitive to hydrothermal conditions in this respect than *H. v. nigricans* and *H. v. rimpaui*.

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