EXPLORING GENETIC AND ENVIRONMENTAL EFFECTS ON NUTRITIONAL AND FUNCTIONAL COMPONENTS IN BARLEY (*HORDEUM VULGARE* L.) GRASS POWDER FOR TWO-YEAR MULTIVARIATE EVALUATION

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(Received 10th Apr 2025; accepted 26th Jun 2025)

Abstract. This study evaluated the genetic variability and environmental influence on nutritional and functional components —flavonoids, γ-aminobutyric acid (GABA), alkaloids, and protein—in grass powder from 43 barley (*Hordeum vulgare* L.) genotypes grown over two consecutive years in Kunming, Yunnan, China. Field trials were conducted in 2021 and 2022 using a randomized complete block design with three replications. Two-year ANOVA revealed highly significant genotypic effects ($p < 0.01$) for all traits, indicating substantial genetic variability. Year effects were significant for flavonoids, GABA, and alkaloids, but not for protein, indicating trait-specific environmental sensitivity. Flavonoid content ranged from 97.69 to 242.24 mg/100 g in year 1 and from 102.38 to 202.29 mg/100 g in year 2. The GABA content increased from 201.75 mg/100 g to 233.62 mg/100 g, while alkaloids contents decreased slightly from 107.82 to 100.49 mg/100 g. Protein content rose marginally from 32.84% to 33.91%. Significant genotype × year interactions for flavonoids and GABA ($p < 0.05$) highlighted trait instability under varying conditions, while protein remained stable across years. Correlation matrix analysis showed strong positive associations between flavonoids and GABA, and moderate correlations with alkaloids, suggesting shared stress-responsive biosynthetic pathways. Protein showed no consistent correlation with secondary metabolites. PCA biplots explained 78.06% (Year 1) and 77.58% (Year 2) of the total variance, effectively discriminating genotypes. Genotypes G41, G11, G3, G7, and G31 exhibited superior trait profiles, while G18, G26, and G34 consistently ranked lowest. Stable genotypes identified in this study provide a foundation for cultivar development aimed at improving human and animal health. These findings provide valuable insight for breeding nutrient-enriched barley cultivars for functional food and feed applications.

Keywords: *barley, genotypes, flavonoids, GABA, alkaloids, protein, nutrition*

DOI: http://dx.doi.org/10.15666/aeer/2305_88638879

Introduction

Barley (*Hordeum vulgare* L.) is one of the earliest domesticated cereal crops and remains a key species globally, both as a food and feed grain and as a raw material for the malt and brewing industries. In recent decades, interest has increased in its potential as a functional food due to the presence of several bioactive compounds, including flavonoids, γ-aminobutyric acid (GABA), alkaloids, and proteins (Shvachko et al., 2021; Khajoane, 2022). These secondary metabolites are not only important for stress tolerance in the plant, but also have significant nutritional and medicinal value for animals and humans. Barley grass, the plant shoot in the pre-seed stage, is especially rich in secondary metabolites and is also becoming popular as a nutritional supplement due to its antioxidant, anti-inflammatory, and neuroprotective activities. Accumulation of these secondary metabolites, however, is highly dependent on genotype and environment and therefore demands rigorous studies in diverse genetic backgrounds and in contrasting time periods (Kobir et al., 2023; Huang et al., 2024).

In the face of climatic variability and increasing consumer demand for health-promoting foods, knowledge of the genotype × environment (G × E) interactions controlling the nutritional composition of barley is paramount. Secondary metabolites like flavonoids and alkaloids are by-products of the phenylpropanoid and nitrogen metabolism pathways that are typically induced under abiotic stress environments. GABA, a non-protein amino acid that is produced in plants through the transformation of glutamate, is associated with a number of plant stress responses and is also the primary neurotransmitter in man. These metabolites are synergistic in the plant defense system and, when taken orally, also bring significant benefits that include maintenance of blood pressure, improved mood, reduced oxidative stress, and potential anti-carcinogenicity (Kiliç et al., 2018; Aklilu et al., 2020).

Although significant, there is limited literature that has assessed co-expression of protein, GABA, flavonoids, and alkaloids among a wide number of barley genotypes under environmental conditions. In addition, while protein is still a prominent nutritional quality in cereals in breeding, protein also has a complex relationship with secondary metabolites and is rarely positively correlated (Karahan and Akgün, 2020; Rehman et al., 2024). Protein composition needs to be understood in relation to how it correlates or fails to correlate with these functional chemicals in the design of multi-nutrient biofortification. It may be difficult to blend top-rated protein genotypes that also embody top-rated secondary metabolites if these traits are in independent or conflicting directions of one another. An analysis tool that addresses the interacting nature of these nutritional characters is therefore needed (Karahan and Akgün, 2020; Qaderi et al., 2023).

Multivariate statistical analysis like genotype-trait Biplot and correlation matrices are also effective tools for the interpretation of inter-trait relationships as well as for the selection of genotypes with desirable nutritional trait combinations. Correlation analysis is useful for revealing underlying physiological relationships between traits, e.g., common biosynthetic precursors or co-regulated pathways. For example, positive correlation of flavonoids with GABA may indicate a coordinated response to oxidative stress or drought stress as seen from earlier studies on wheat and rice (Ibrahim et al., 2019; Huang et al., 2024). Alkaloids and flavonoids also share common metabolic precursors like phenylalanine and are regulated by overlapping transcriptional networks (Shvachko et al., 2021; Verma et al., 2022; Yang et al., 2023, 2025). Biplot also helps to reduce the dimensionality of multivariate data sets so that genotype performance can be displayed across a number of traits and the main sources of variation determined. By looking at trait loadings and positions of genotypes in multivariate space, researchers can screen efficiently for genotypes with superior or divergent nutritional profiles (Massman et al., 2022; Yang et al., 2023).

Temporal replication over a number of years is necessary in such research to account for environmental impacts, particularly as these phytochemical pathways are reported to be affected by abiotic stress factors such as temperature, drought, salinity, and soil fertility. Year-to-year variation allows for the separation of stable and plastic genotypes, informing breeding decisions suitable for different agro-ecological environments. Moreover, screening genotypes across contrasting years also enhances the coherence of associations and the selection accuracy of the top genotypes for functional food breeding (Kobir et al., 2023; Patial et al., 2023). In this context, this research tested nutritional and functional metabolites of 43 genotypes of barley during two successive seasons using both correlation-based and Biplot-based approaches to identify highly performing and stable genotypes.

Although previously there were individual studies on protein or secondary metabolites in barley or other cereals, there were few studies that had a comprehensive approach and also examined them in a pool of genetic variability in combination. In addition, the identification of stable year-to-year associations, particularly between years that had diverse environmental stimuli, provides valuable inputs on the heritability of traits as well as potential gene linkage (Ibrahim et al., 2019). It not only provides easier functional enhancement in barley cultivars by breeding, but also provides opportunities for marker-assisted gene selection of secondary metabolite synthesis pathway genes. Unlike earlier studies that focused on either a single trait, one-year trials, or a limited set of genotypes, the present research provides a novel and comprehensive perspective by combining multivariate statistics with two-season evaluations across 43 genetically diverse barley genotypes. By analyzing genotype × year interactions for flavonoids and GABA alongside trait interrelationships using correlation matrices and PCA biplots, this study uncovers patterns of trait stability and variability under changing environmental conditions. This level of integration is rare in barley research, particularly in the context of secondary metabolite enrichment in grass powder form (Kobir et al., 2023; Patial et al., 2023). Our findings thus offer new insights into selecting genotypes with stable and synergistic nutritional profiles, enhancing the value of barley as a functional food and feed crop. The results also inform future breeding programs aiming to develop climate-resilient, nutrient-rich barley cultivars.

The objectives of this study were threefold: (1) to quantify the levels of key nutritional and functional components—namely flavonoids, γ-aminobutyric acid (GABA), alkaloids, and protein—in barley grass powder derived from 43 genotypes across two growing seasons; (2) to evaluate interrelationships among these traits using correlation matrix analysis to explore underlying biological and metabolic linkages; and (3) to rank and classify genotypes using multivariate tools, including PCA biplots, to identify stable, high-performing candidates with desirable nutritional profiles. This integrative approach combines biochemical measurement, trait interdependence analysis, and genotype performance visualization to guide future breeding strategies for functional food and feed applications.

Materials and methods

Experimental materials and field design

The experimental materials (*Table 1*) comprised 43 barley (*Hordeum vulgare* L.) genotypes provided by the Institute of Biotechnology and Germplasm Resources, Yunnan Academy of Agricultural Sciences, Kunming, Yunnan, China. These genotypes included malt barley, feed barley, and hulless barley types. Field trials were conducted at the experimental research site in Dianyuan Town, Panlong District, Kunming City (elevation: 1,973 meters). The site was characterized by moderately fertile soil.

Table 1. *List of the 43 barley genotypes used in the study, along with their corresponding code, name, type (beer, feed, or hulless), and row type (two-rowed or six-rowed)*

Code	Name	Type	Row	Code	Name	Type	Row
G1	Yunpi 9	Malt Barley	2	G23	Yunjing 2	Beer Barley	2
G2	Yunpi 10	Malt Barley	2	G24	Yunpi 17	Malt Barley	2
G3	Yunpi 11	Malt Barley	2	G25	Yunsimai 7	Feed Barley	6
G4	Yunsimai 1	Feed Barley	2	G26	Yunsimai 8	Feed Barley	6
G5	Yunsimai 2	Feed Barley	6	G27	Yunpi 18	Malt Barley	2
G6	Yunsimai 3	Feed Barley	6	G28	Yunpi 20	Malt Barley	2
G7	Aisigan 4	Malt Barley	2	G29	Tengyunmai 4	Malt Barley	2
G8	Yunpi 7	Malt Barley	2	G30	Yunsimai 9	Feed Barley	6
G9	Yunpi 5	Malt Barley	2	G31	Yunsimai 10	Feed Barley	6
G10	Yanmi 2	Malt Barley	2	G32	Yunpi 21	Malt Barley	2
G11	Auxuan 3	Malt Barley	2	G33	Yunpi 22	Malt Barley	2
G12	Yunpi 2	Malt Barley	2	G34	Yunsimai 11	Feed Barley	6
G13	Yunpi 4	Malt Barley	2	G35	Yunsimai 12	Feed Barley	6
G14	Yunke 4	Hulless Barley	6	G36	Yunpi 3	Malt Barley	2
G15	Zheyun 1	Malt Barley	2	G37	Yunsimai 13	Feed Barley	6
G16	Kunpi 2	Malt Barley	2	G38	Yunsimai 14	Feed Barley	6
G17	Yunsimai 4	Feed Barley	6	G39	Kunpi 4	Malt Barley	2
G18	Yunpi 12	Malt Barley	2	G40	Asi82gan1	Malt Barley	2
G19	Yunpi 14	Malt Barley	2	G41	Yunpi 24	Malt Barley	2
G20	Yunyu 1	Malt Barley	2	G42	Yungong 1	Malt Barley	2
G21	Yunke 1	Hulless Barley	6	G43	Yungong 2	Malt Barley	2
G22	Yunpi 15	Malt Barley	2				

The experiment was conducted in a three-replicated randomized complete-block design (RCBD). Three rows of each genotype were sown and 200 seeds were given to each. Each row was 2 meters in length and there was a 0.3-meter row-to-row spacing. Strip sowing was practiced and regular agronomic practices were followed. Experiments were conducted for two successive autumn seasons with sowing dates of November 1, 2021, and November 1, 2022, respectively. Thinning of the seedlings was carried out in the jointing stages. After harvesting, plant material from all treatments in the experiments was air dried, ground, packaged, and then stored in −20°C for future analysis.

Determination of nutritional and functional component contents in barley

The determinations of nutritional and functional components—total flavonoids, γ-aminobutyric acid (GABA), alkaloid, and protein contents—were carried out in the Key Laboratory of the Institute of Biotechnology and Germplasm Resources of Yunnan Academy of Agricultural Sciences. Barley grass powders were analyzed in triplicate (three biological replicates) and mean values were obtained. Flavonoid was analyzed by using a method of aluminum nitrate colorimetric according to the approach of Zhao et al. (2011). GABA content was measured following the method of (Inatomi and Slaughter, 1975). Alkaloid content was quantified using the method described by Han (2010). Protein content was estimated using the Kjeldahl method. For protein estimation, 1.0000 ± 0.0001 g of the powdered sample was placed into a 250 mL digestion tube. Two Kjeldahl catalyst tablets (equivalent to 7 g K_2SO_4 and 0.8 g $CuSO_4{\cdot}5H_2O$) and 12 mL of concentrated H_2SO_4 were added. Digestion was performed at 420°C for 2 hours using an 8400 Automatic Kjeldahl Nitrogen Analyzer. After cooling, titration was conducted, and the titrant concentration was accurate to four decimal places. A blank test was included with each batch of samples. Protein content (%) was calculated using the formula:

$$\text{Protein (\%)} = \text{Nitrogen (\%)} \times 6.25 \qquad \text{(Eq.1)}$$

where:
T = Volume (mL) of HCl used in sample titration,
B = Volume (mL) of HCl used in blank titration,
N = Normality of HCl (accurate to four decimal places).

Statistical analysis

Data were analyzed using Microsoft Excel 2019 and IBM SPSS Statistics 26. The mean values from three biological replicates were used to compute the content of each functional component. One-way analysis of variance (ANOVA) was performed following the method of Steel et al. (1997). Pearson correlation analysis was conducted to evaluate relationships among the traits, and genotype-by-trait biplot analysis was carried out to visualize genotype performance and trait interactions.

Results and discussion

The analysis of variance (ANOVA) revealed significant main effects of genotype ($p < 0.01$) on all four traits—flavonoids, GABA, alkaloids, and protein—indicating considerable genetic variation among the 43 barley genotypes. The year effect was also significant for flavonoids and GABA ($p < 0.01$), and for alkaloids ($p < 0.05$), suggesting that these components are environmentally responsive. In contrast, the year effect for protein was not significant, indicating relative stability of this trait across seasons. Regarding genotype × year interactions, significant effects were observed only for flavonoids and GABA ($p < 0.05$), suggesting differential genotypic responses to environmental variation for these traits. No significant interactions were found for alkaloids and protein, supporting the stability of their expression across years. These findings highlight the importance of both genetic and environmental contributions to nutritional trait variation and underscore the relevance of multi-season evaluation for identifying stable, high-performing genotypes.

The significant genotype × year interactions observed for flavonoids and GABA indicate that these traits are influenced not only by genotype and environment independently but also by their interaction. This underscores the presence of trait plasticity, where certain genotypes respond differently to environmental variation over time. From a breeding perspective, these interactions are critical: they help differentiate stable genotypes—those with consistent trait expression—from plastic genotypes, which may show enhanced accumulation of bioactive compounds under specific environmental stimuli. For example, the consistent performance of genotypes such as G41 and G31 across years for GABA suggests high genetic stability, while others showing year-specific peaks may be valuable for targeted environments. Thus, understanding G × Y interactions provides essential guidance for developing barley varieties tailored to variable climates or specific functional food markets, where either stability or responsiveness may be desired traits.

Table 2 Analysis of Variance (ANOVA) displayed the differences among the nutritional functional components of barley grass powder (flavonoids, γ-aminobutyric acid (GABA), alkaloids, and protein) of 43 barley genotypes in two years. Genotypic effect was significant in each of the four components ($p < 0.01$), and genetic differences among the barley genotypes significantly contributed to the nutritional value of the barley grass powder (Khajoane, 2022; Massman et al., 2022). This suggested that genetic selection can be used to improve the levels of these bioactive compounds in barley. The year effect was also highly significant for flavonoids, and GABA ($p < 0.01$), and significant for alkaloids ($p < 0.05$), indicating that environmental factors, including growing conditions such as climate and soil quality, played a major role in influencing the nutritional composition of barley across the two years. The year effect for protein content was non-significant which indicating that protein contents not effected by environmental factor and remained stable. Such environmental variation has been previously observed in other studies on bioactive components in crops (Pellegrino et al., 2022; Verma et al., 2022; Fatemi et al., 2023), confirming the significant role of temporal conditions on the expression of nutritional traits.

Table 2. *Mean squares from analysis of variance (ANOVA) for nutritional and functional components in barley grass powder, based on two years of data from 43 barley genotypes*

Sources of Variation	**DF**	**Flavonoids**	**γ-aminobutyric acid**	**Alkaloids**	**Protein**
Replication	2	34.25	4.53	6.89	2.09
Genotypes	42	25.83**	13.98**	15.72**	87.64**
Years	1	19.42**	31.10**	2410.57*	9125.8ns
Genotypes*Years	42	12.01*	17.98*	8.33ns	51.27ns
Error	170	2.73	3.29	2.07	1.62
Total	257				

**= highly significant at 0.01, *= significant at 0.05, ns= non-significant

The interaction between genotypes and years was found to be significant for flavonoids and GABA ($p < 0.05$), indicating that the genotypic performance of these components can vary across different growing seasons. This suggests that certain genotypes might be more adaptable to environmental fluctuations, resulting in consistent production of flavonoids and GABA. In contrast, the genotypes × years interaction was not significant for alkaloids and protein, indicating that the performance of barley genotypes for these two components remained stable across different years, irrespective of environmental changes. This finding could be crucial for breeding programs aiming to develop barley cultivars with consistent nutritional profiles. The significant variation observed for both genotypes and years underlines the importance of considering both genetic potential and environmental conditions when evaluating and selecting barley genotypes for nutritional improvement (Fan et al., 2023; Kobir et al., 2023; Ijaz et al., 2023).

ANOVA revealed a significant genotype effect for all the characters, showing that there was considerable genetic variability among the 43 barley lines. Year had a significant influence on GABA and the contents of the flavonoids, proving the role of the environment. Year × genotype interaction was significant in GABA and the flavonoids, proving differential stability of characters across years. Protein content was, on the contrary, fairly stable across years and genotypes, and there were no interacting effects that were significant. These findings align with previous research indicating the complex interplay between genetic factors and environmental conditions in shaping the nutritional and functional components in crops (Patial et al., 2023; Maziriri et al., 2024; Hejazi et al., 2025). The results of this ANOVA support the potential for breeding programs that target specific nutritional profiles, particularly for bioactive components like flavonoids, GABA, alkaloids, and protein, which are of significant interest for human health and functional food applications.

Characterization of genotypes in different years using studied traits

Flavonoids

The flavonoid content among barley genotypes showed notable variation across both years. In year 1, the flavonoid content ranged from 97.69 to 242.24 mg/100 g, with an average of 160.02 mg/100 g (*Table 3*). In contrast, year 2 showed a slightly narrower range (102.38 to 202.29 mg/100 g) and a reduced mean value of 145.91 mg/100 g. The higher variance (1481.28) and standard deviation (29.49) in year 1 indicate broader genotypic diversity or a greater environmental influence compared to year 2. The coefficient of variation (CV) also declined slightly from 18.43% to 17.56%, suggesting relatively stable expression across genotypes despite year-to-year fluctuations. These results are in agreement with (Ndlovu et al., 2024; Paire et al., 2024), who emphasized that flavonoid biosynthesis in barley is influenced by environmental cues such as light intensity and temperature, which may have varied between years.

Table 3. *Descriptive statistics of nutritional and functional components in barley grass powder from 43 barley genotypes evaluated over two consecutive years*

Parameters	Season	Flavonoids (mg/100g)	γ-aminobutyric acid (mg/100g)	Alkaloids (mg/100g)	Protein (%)
Minimum	Year 1	97.69	133.01	91.77	29.02
	Year 2	102.38	191.14	74.49	30.90
Maximum	Year 1	242.24	314.61	123.37	35.12
	Year 2	202.29	290.72	128.42	39.29
Average	Year 1	160.02	201.75	107.82	32.84
	Year 2	145.91	233.62	100.49	33.91
Variances	Year 1	1481.28	2552.53	79.89	2.21
	Year 2	656.70	661.34	193.87	3.51
Standard Deviation	Year 1	29.49	33.52	8.94	1.49
	Year 2	25.63	25.72	13.92	1.87
Coefficient of Variation	Year 1	18.43	16.62	8.29	4.52
	Year 2	17.56	11.01	13.86	5.53

The performance of barley genotypes in terms of flavonoid content varied significantly between the two years (*Table 4*). In Year 1, the top-performing genotypes for flavonoids were G31 (242.24 mg/100 g), G7 (239.84 mg/100 g), and G41 (220.84 mg/100 g), while the lowest were G18 (97.69 mg/100 g), G26 (107.41 mg/100 g), and G39 (108.07 mg/100 g). The highest flavonoid concentrations in Year 2 were found in G31 (178.3 mg/100 g), G3 (179.3 mg/100 g), and G11 (181.48 mg/100 g), with the lowest genotypes again being G18 (102.38 mg/100 g), G26 (106.35 mg/100 g), and G39 (106.35 mg/100 g) as mentioned in *Table 4*. High flavonoid genotypes such as G31 and G7 may be used to develop barley varieties with enhanced antioxidant capacities, offering potential health benefits. This finding aligns with previous research by Paire et al. (2025), and Yang et al. (2025), who highlighted the genetic potential for improving flavonoid content in barley. The significant variation between genotypes suggests that genetic diversity is a key factor in maximizing the nutritional value of barley. Barley grass powder, rich in flavonoids, serves as a natural antioxidant supplement. In humans, these compounds reduce oxidative stress, lower inflammation, and have protective effects against cardiovascular diseases and certain cancers (Huang et al., 2024). In animals, flavonoid supplementation has shown to improve immune response, reduce gut inflammation, and enhance overall health and productivity (Shvachko et al., 2021). Therefore, flavonoid-rich barley grass can be utilized in functional foods and feed formulations to promote health in both humans and livestock.

Table 4. *Top five and bottom five performing barley genotypes for nutritional and functional components in barley grass powder during Year 1 and Year 2, selected from a total of 43 genotypes*

Nutritional Components	Seasons	Best Performer Genotypes with their mean values	Lowest Performer Genotypes with their mean values
Flavonoids (mg/100g)	Year 1	G41 (220.84), G11 (223.86), G3 (227.97), G7 (239.84), G31 (242.24)	G18 (97.69), G26 (107.41), G39 (108.07), G34 (111.06), G12 (111.62)
	Year 2	G31 (178.3), G3 (179.3), G11 (181.48), G41 (200.7), G7 (202.29),	G18 (102.38), G26 (106.35), G39 (106.35), G12 (106.45), G34 (107.74)
γ-aminobutyric acid (mg/100g)	Year 1	G11 (282.63), G3 (295.71), G7 (297.63), G31 (299.03), G41 (314.61)	G39 (133.01), G18 (134.44), G34 (139.15), G12 (140.78), G26 (146.07)
	Year 2	G20 (270.65), G11 (275.39), G41 (280.02), G7 (290.72), G31 (290.72),	G34 (191.14), G39 (192.74), G12 (198.69), G18 (201.39), G26 (202.01),
Alkaloids (mg/100g)	Year 1	G41 (121.25), G31 (122.45), G25 (122.55), G7 (122.94), G3 (123.37)	G13 (91.77), G37 (95.78), G12 (95.99), G18 (96.29), G39 (96.45)
	Year 2	G31 (117.27), G8 (117.95), G41 (121.38), G3 (124.8), G7 (128.42)	G22 (74.49), G30 (76.95), G34 (78.39), G12 (79.14), G18 (79.63)
Protein (%)	Year 1	G7 (34.73), G28 (34.75), G26 (34.76), G11 (35.06), G41 (35.12)	G5 (29.02), G30 (30.33), G16 (30.46), G34 (30.63), G12 (30.89)
	Year 2	G11 (35.64), G41 (36.09), G31 (37.79), G7 (38.01), G8 (39.29)	G34 (30.9), G42 (31.01), G12 (31.08), G16 (31.17), G26 (31.34)

γ-Aminobutyric Acid (GABA)

GABA levels varied significantly between the two years. The average GABA content increased from 201.75 mg/100 g in year 1 to 233.62 mg/100 g in year 2, with the minimum and maximum values also rising (*Table 3*). Although the variance was higher in year 1 (2552.53), the coefficient of variation decreased from 16.62% to 11.01% in year 2, reflecting a more uniform distribution of GABA content among genotypes in the latter season. This reduction in variability may indicate that specific environmental conditions in year 2 favored consistent expression of GABA biosynthesis genes. These results are in agreement with Shvachko et al. (2021), who observed that environmental stress, such as limited water availability or temperature shifts, enhances GABA accumulation in cereal grasses. In Year 1, the highest concentrations of GABA were found in genotypes G41 (314.61 mg/100 g), G31 (299.03 mg/100 g), and G7 (297.63 mg/100 g), while the lowest concentrations were observed in G39 (133.01 mg/100g), G18 (134.44 mg/100g), and G34 (139.15 mg/100 g). The Year 2 results were similar, with G20 (270.65 mg/100g), G11 (275.39 mg/100 g), and G41 (280.02 mg/100 g) showing the highest GABA content, while G34 (191.14 mg/100 g), G39 (192.74 mg/100 g), and G12 (198.69 mg/100 g) had the lowest (*Table 4*).

The two high GABA-containing genotypes G41 and G31 have the potential to be used as candidate breeding GABA-enriched barley cultivars for human health promotion. This is consistent with the work of Rehman et al. (2024), who determined that stress could be managed and mental well-being enhanced through heightened GABA intake via functional foods. Moreover, in animals, GABA supplementation has been linked with better gut health and greater feed conversion efficiency (Yang et al., 2013), implicating the wider application of barley grass powder as a functional feed ingredient. GABA from barley grass powder is widely known to possess neuroprotective activity. In humans, it correlates with reduced anxiety, improved sleep, and improved regulation of blood pressure (Ramakrishna et al., 2019). In animal diets, GABA supplementation enhances the feed efficiency, resistance to stress, and neurological growth in poultry, swine, and fish species (Qaderi et al., 2023). Thus, barley grass powder fortified with GABA is a favorable ingredient in animal feed premixes and nutraceuticals.

Alkaloids

Alkaloid content showed distinct variation across the years, with a slight reduction in average levels from 107.82 mg/100 g in year 1 to 100.49 mg/100 g in year 2 (*Table 3*). Interestingly, while the mean decreased, the variance and standard deviation increased notably in year 2 (193.87 and 13.92, respectively), indicating greater genotypic diversity or stronger environmental effects on alkaloid biosynthesis. The coefficient of variation also increased from 8.29% in year 1 to 13.86% in year 2. This shift may suggest a differential genotypic response to changing environmental conditions, as alkaloids are often produced in higher quantities under stress for plant defense (Guo et al., 2024). Thus, selecting genotypes with stable alkaloid levels across years could be crucial for nutritional and pharmaceutical applications of barley. The Year 1 data revealed that the genotypes G41 (121.25 mg/100 g), G31 (122.45 mg/100 g), and G25 (122.55 mg/100 g) had the highest levels of alkaloids, while G13 (91.77 mg/100 g), G37 (95.78 mg/100 g), and G12 (95.99 mg/100 g) had the lowest concentrations. In Year 2, G31 (117.27 mg/100 g), G8 (117.95 mg/100 g), and G41 (121.38 mg/100 g) were the top performers, while G22 (74.49 mg/100 g), G30 (76.95 mg/100 g), and G34 (78.39 mg/100 g) showed the lowest alkaloid concentrations (*Table 4*).

Alkaloids are bioactive compounds that contain a wide range of therapeutic activities, including antimicrobial, anticancer, and analgesic activities. However, they are toxic when used in excess amounts and hence have to be regulated strictly. Genotypes G41 and G31 with enhanced alkaloid content can be employed for the development of barley types with controlled amounts of alkaloids for functional food applications. Barley enriched with alkaloids has also been discovered to possess antimicrobial activities, and these might prove useful in the welfare of animals, as attested by Shvachko et al. (2021), who reported increased immune ability in cattle fed alkaloid-enriched barley.

Protein

Protein content in barley genotypes remained relatively stable but exhibited a slight increase in year 2 (33.91%) compared to year 1 (32.84%). The minimum and maximum values also increased, suggesting an overall improvement in grain protein accumulation (*Table 3*). Notably, both variance and standard deviation increased in year 2 (3.51 and 1.87, respectively), indicating broader diversity. The coefficient of variation increased from 4.52% to 5.53%, reflecting higher relative variability among genotypes. These changes may result from environmental differences affecting nitrogen uptake and assimilation pathways. Previous studies by Yang et al. (2023) have highlighted the sensitivity of grain protein content to agronomic conditions such as soil fertility, temperature, and rainfall, which could have contributed to the observed differences. Similar trends were reported by Huang et al. (2024), noting that protein accumulation in barley is highly dependent on genotype and soil nutrition.

Protein content in barley varied significantly between genotypes. In Year 1, the genotypes G7 (34.73%), G28 (34.75%), and G26 (34.76%) exhibited the highest protein levels, while the lowest were G5 (29.02%), G30 (30.33%), and G16 (30.46%) as shown in *Table 4*. The Year 2 data showed G11 (35.64%), G41 (36.09%), and G31 (37.79%) as the top performers, while G34 (30.9%), G42 (31.01%), and G12 (31.08%) had the lowest protein content. Protein is an essential macronutrient that plays a crucial role in muscle development, tissue repair, and metabolic functions. The genotypes with higher protein content, such as G7 and G31, offer great potential for improving the nutritional quality of barley, which can be used to produce high-protein barley flour or barley-based functional foods. This aligns with previous findings by Maziriri et al. (2024), and Rehman et al. (2024), who emphasized the importance of barley as a source of digestible protein for both human consumption and animal feed. In livestock, barley protein enhances growth performance and feed utilization (Baloch et al., 2024; Yang et al., 2025), supporting the role of protein-rich barley in improving animal health and productivity.

Correlation matrix

In the present study, *Figure 1* presents correlation matrices for the nutritional functional components extracted from barley grass powder across 43 genotypes, displayed separately for Year 1 (*Figure A*) and Year 2 (*Figure B*). The correlation matrices revealed statistically significant associations among flavonoids, GABA, and alkaloids across both years. Notably, flavonoids and GABA exhibited a strong positive correlation ($r = 0.62$ in Year 1; $r = 0.54$ in Year 2), while moderate correlations were also observed between flavonoids and alkaloids, and between GABA and alkaloids. This relationship is supported by the fact that both compounds are part of stress-responsive metabolic pathways and play key roles in antioxidative defense and metabolic regulation (Al-Tabbal and Al-Fraihat, 2012; Dyulgerova, 2012; Ramakrishna et al., 2019; Aklilu et al., 2020). These associations suggest a possible coordinated expression under similar environmental conditions. While such trends may imply physiological or stress-related links, we interpret these relationships cautiously, recognizing that correlation does not confirm a shared biosynthetic origin. Although previous studies have reported overlapping precursor pathways or environmental triggers for these compounds, our study does not include molecular-level validation. Therefore, we present these correlations as useful for phenotype-based selection and hypothesis generation rather than conclusive evidence of biochemical linkage. Future studies incorporating gene expression or metabolomic profiling would be necessary to verify underlying regulatory mechanisms.

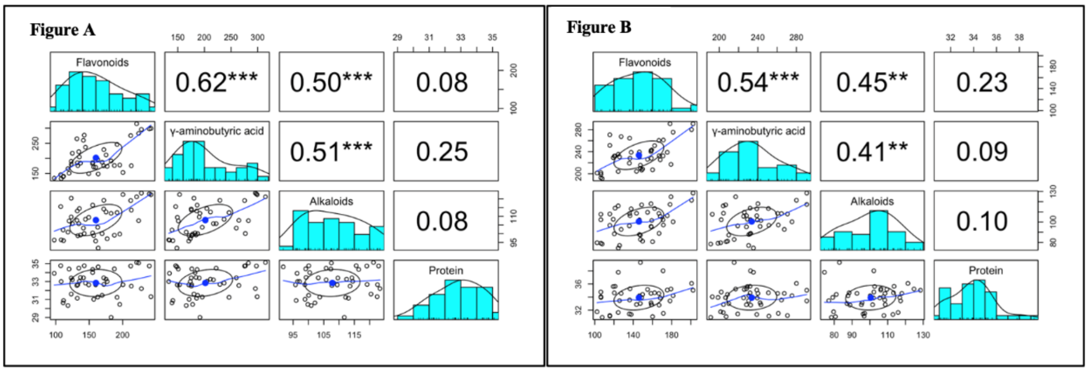

Figure 1. *Correlation matrix among nutritional and functional components in barley grass powder derived from 43 barley genotypes during Year 1 (A) and Year 2 (B). Significance levels are indicated as follows: *** = highly significant at* $p < 0.01$*, ** = significant at* $p < 0.05$*, ns = non-significant*

Similarly, the positive correlation between flavonoids and alkaloids (r = 0.50** in Year 1; r = 0.45** in Year 2) suggests a coordinated regulation of phenolic and nitrogenous secondary metabolites. This observation is consistent with the findings of Pellegrino et al. (2022), and Huang et al. (2024), who reported that phenolic and alkaloid compounds often share precursor pathways and exhibit synergistic roles in plant defense mechanisms. GABA and alkaloids also demonstrated a consistent significant correlation across both years (r = 0.51*** and r = 0.41**, respectively), which may reflect the involvement of glutamate-derived pathways in the biosynthesis of both compounds. The histograms along the diagonal depict the distribution of each component, providing insight into their variability across the genotypes. The mean values for these components in Year 1, as indicated in the scatterplots, suggest that the average γ-aminobutyric acid content is around 150-300 (mg/100 g), while the average Alkaloids content is approximately 95-115 (mg/100 g). In Year 2, the mean Alkaloids content, around 80-130 (mg/100 g), while the mean γ-aminobutyric acid content is around 200-280 units. GABA is synthesized from glutamate, and many alkaloids also originate from amino acid-based precursors, hinting at a potential metabolic link (Yang et al., 2013; Ramakrishna et al., 2019).

Protein content, however, displayed year-dependent variability in its association with other traits. In Year 1, protein was not significantly correlated with flavonoids (0.08), GABA (0.25), or alkaloids (0.08), indicating an independent regulatory mechanism possibly driven by genetic factors. In Year 2, protein showed also non-significant positive correlations with flavonoids (r = 0.23), GABA (r = 0.09), and alkaloids (r = 0.10). This change may be attributed to environmental variations affecting nitrogen assimilation and its allocation to both primary (protein) and secondary metabolites. The histograms along the diagonal depict the distribution of mean values, providing insight into their variability across the genotypes (Ibrahim et al., 2019). The mean protein content remains relatively similar to both years, around 29-38 % in the present study.

The strong positive correlations of flavonoids, γ-aminobutyric acid, and alkaloids between the two years suggest a potential synergistic interaction or shared regulating mechanisms of these factors in barley grass. The absence of high correlation of protein shows that its accumulation is likely to be regulated by independent factors. The same findings were made by Al-Tabbal and Al-Fraihat (2012), Dyulgerova (2012), and Yang et al. (2013), who highlighted the role of environmental control of protein and secondary metabolite biosynthesis in cereal crops. This validates the decisive role of genotype × environment interaction in determining nutritional quality and the strategic decision of selecting genotypes possessing stable and enriched functional profiles. These results are of high importance for breeding programs that focus on strengthening the nutraceutical potential of barley (Massman et al., 2022; Maziriri et al., 2024).

Biplots

Figure 2 illustrated the biplots based on nutritional functional components—namely protein, alkaloids, γ-aminobutyric acid, and flavonoids—extracted from barley grass powder among 43 barley genotypes evaluated over two consecutive years. The biplots (*Figure 2A* for Year 1 and *Figure 2B* for Year 2) reveal the distribution of genotypes in the multivariate space defined by the first two principal components (PC1 and PC2), which together explain a substantial proportion of the total variance in the data. In Year 1 (*Figure 2A*), PC1 accounted for 53.53% and PC2 for 24.53% of the total variation, summing to 78.06%. In contrast, in Year 2 (*Figure 2B*), PC1 and PC2 contributed 61.76% and 15.83%, respectively, amounting to a cumulative variance of 77.58%. This indicates that the first two principal components captured the majority of the variability among the genotypes in both years. In both Year 1 (*Figure A*) and Year 2 (*Figure B*), the vectors representing these components reveal consistent trends, despite slight variations in the explained variance (Aklilu et al., 2020; Shvachko et al., 2021; Kobir et al., 2023; Maziriri et al., 2024). Notably, protein and γ-aminobutyric acid vectors consistently point towards the upper right quadrants, indicating a positive correlation with the principal components capturing the majority of the variance, suggesting that genotypes located in these regions, such as G8 and G41, tend to exhibit higher levels of these components across both years. Conversely, the flavonoid vector is consistently directed towards the lower right quadrants, signifying that genotypes like G31, positioned in this region, are characterized by elevated flavonoid content throughout both experimental periods. The alkaloid vector exhibits some variability in direction, particularly between the two years, suggesting a potentially higher sensitivity to environmental or year-specific influences; however, it generally contributes to the separation of genotypes along the first principal component (Pellegrino et al., 2022; Verma et al., 2022; Kobir et al., 2023).

The distribution of genotypes on the biplots indicates significant variation in nutritional content. The genotypes located closer to the center of the biplots have comparatively mean levels of the factors measured, whereas the others that are far from the center have extreme values, indicating specific nutritional characteristics. Protein content was highly positively loaded on PC2 during Year 1, clearly distinguishing between genotype G28, being well down the protein vector direction and having the highest concentration of protein among the genotypes. Conversely, alkaloids, γ-aminobutyric acid, and flavonoids loaded heavily on PC1, forming a cluster of correlated traits (Kiliç et al., 2018; Ibrahim et al., 2019; Karahan and Akgün, 2020). Genotypes such as G3, G31, and G45 exhibited high positive scores along PC1, implying elevated concentrations of these secondary metabolites. The majority of the remaining genotypes were located near the origin of the biplot, suggesting average or balanced levels of all the measured nutritional components. This distribution reflects substantial variability among the genotypes, particularly in secondary metabolites, while indicating moderate profiles for most genotypes (Güngör et al., 2024). In Year 2, the pattern of trait associations was generally consistent with Year 1, though there were changes in the position of genotypes and loadings of traits. Protein and alkaloid vectors remained salient and well differentiated, indicating the significant influence that the two had on genotype differentiation. Genotypes G7, G31, and G41 were in the direction of protein and alkaloid vectors, which indicated that these genotypes were well supplied with these chemicals. Also, genotypes G3 and G11 that in Year 1 were strongly associated with γ-aminobutyric acid and flavonoids, in Year 2 had the same association, indicating temporal consistency of these nutritional traits. Replication of clustering of traits and relative positions of certain genotypes in the two years indicates the strength and potential of certain genotypes for nutritional improvement (Kendal et al., 2019; Fan et al., 2023; Güngör et al., 2024).

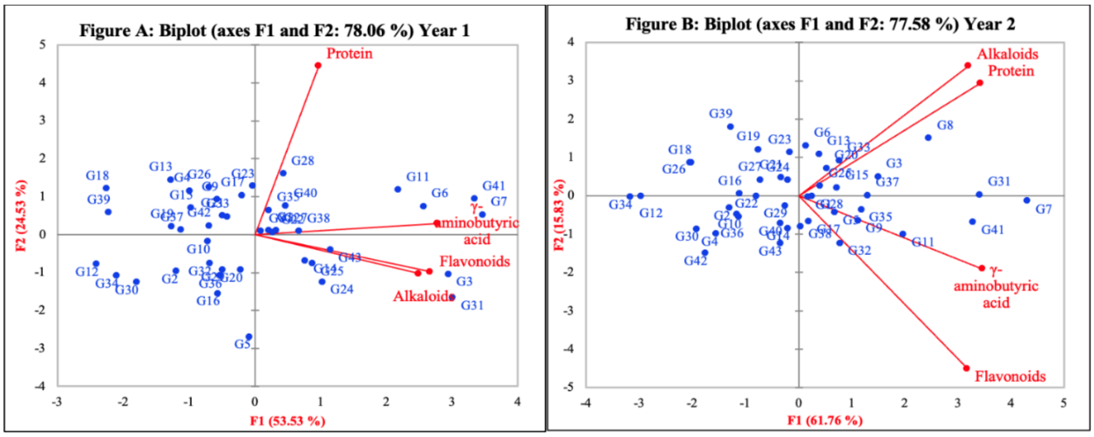

Figure 2. *Genotype-by-trait biplot illustrating the relationships among nutritional and functional components extracted from barley grass powder in 43 barley genotypes during Year 1 (Figure A) and Year 2 (Figure B)*

Notably, several genotypes consistently exhibited superior performance in most of the studied traits. Specifically, G41, G11, G3, G7, and G31 demonstrated consistently high levels of the measured nutritional components during both years. These genotypes, characterized by their robust performance, hold significant potential for inclusion in breeding programs aimed at enhancing the nutritional quality of barley. Conversely, a subset of genotypes consistently displayed the lowest levels of the studied components. Genotypes G18, G26, G39, G34, and G12 consistently exhibited the poorest performance, indicating limited potential for nutritional enhancement (Karahan and Akgün, 2020; Khajoane, 2022). The consistent trends observed in both years underscore the reliability of these findings and highlight the potential for targeted selection of superior genotypes for breeding purposes. The identification of both high-performing and low-performing genotypes provides valuable insights for developing barley varieties with improved nutritional profiles. The biplots therefore provide a comprehensive overview of the relationships between nutritional components and barley genotypes, facilitating the identification of genotypes with desirable nutritional profiles for breeding programs (Taheripourfard et al., 2017; Aklilu et al., 2020; Pellegrino et al., 2022). The high protein, flavonoid, and GABA content observed in specific genotypes suggests significant potential for improving human and animal health. Future research should focus on further characterizing the specific compounds present, optimizing extraction methods, and conducting clinical trials to validate the observed benefits.

The nutritional traits evaluated in this study—flavonoids, GABA, alkaloids, and protein—are intended for improvement in both human food products and animal feed formulations. Flavonoids and GABA contribute to antioxidant activity, blood pressure regulation, and neurological health in humans, while also enhancing immune response and stress tolerance in animals. High protein content is vital for muscle development and metabolic function across both consumer groups. However, traits such as alkaloids, while offering antimicrobial benefits, must be carefully monitored due to their potential toxicity at elevated levels, especially in human food applications (Karahan and Akgün, 2020; Khajoane, 2022; Kadege et al., 2024). Therefore, breeding programs should aim for genotypes with high nutritional value, functional efficacy, and controlled levels of potentially bioactive compounds. This dual-purpose approach ensures the safe and effective use of barley grass powder as a functional ingredient in health-oriented food systems and nutritionally optimized animal diets.

Conclusions

This study comprehensively assessed 43 barley genotypes over two growing seasons to elucidate the genetic and environmental influences on key nutritional and functional components. ANOVA confirmed significant genotypic effects ($p < 0.01$) on flavonoids, GABA, alkaloids, and protein, underscoring the strong genetic basis for these traits. Environmental variation particularly influenced flavonoid and GABA accumulation, while protein content remained relatively stable. Genotypes G31 and G7 exhibited high flavonoid content, suggesting strong antioxidant potential. GABA levels increased significantly in Year 2, reflecting responsiveness to environmental cues; genotypes G41 and G31 maintained high GABA across both years, indicating genetic consistency. Alkaloid content showed year-specific variability, requiring careful consideration in breeding for pharmaceutical applications. Protein content, ranging from 29.02% to 37.79%, showed modest variation and was highest in G31 and G41, identifying them as nutritionally superior. Strong correlations between flavonoids and GABA ($r = 0.62^{***}$, 0.54^{***}) and moderate associations with alkaloids ($r \approx 0.45–0.50^{**}$) suggest shared stress-responsive biosynthetic pathways. Protein content exhibited no significant correlation with secondary metabolites, indicating independent regulation. PCA biplots explained over 77% of the variance in both years and revealed distinct genotype clustering. Genotypes G41, G3, and G31 showed consistently superior trait profiles, while G18 and G26 ranked lowest. Significant genotype × year interactions for flavonoids and GABA emphasize the importance of multi-environment testing. These findings offer a robust foundation for targeted breeding of nutritionally enriched barley. Multivariate and correlational analyses provide essential tools for cultivar development, enhancing barley's value as a functional food and feed crop.

Funding. This research work was supported by the China Agriculture Research System of MOF and MARA (CARS-05-01A-04) China.

Conflict of interest. The authors have no conflicts of interest.

Data availability statement. All relevant data are included in the manuscript file.

Acknowledgment. The authors extend their appreciation to the Researchers Supporting Project number (RSPD2025R1048), King Saud University, Riyadh, Saudi Arabia.

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