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Genetic and Environmental Variation in Health-Promoting L-Homoarginine and Neurotoxic β -ODAP in Grass Pea (*Lathyrus sativus* L.)

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ABSTRACT

Grass pea (*Lathyrus sativus* L.) is a promising legume crop due to its resilience to climatic stress factors such as drought and waterlogging. However, the occurrence of the neurotoxin β -N-oxalyl-L- α , β -diaminopropionic acid (β -ODAP) and the lack of adapted varieties pose significant challenges for its wider adoption across Europe. On the other hand, the presence of L-homoarginine (L-hArg) in grass pea has gained attention for its benefits on cardiovascular health. In this study, contrasting grass pea genotypes were grown at three locations in Germany, and the concentrations of α -, β -ODAP, L-hArg, and 17 other free amino acids were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS). β -ODAP concentrations ranged from 0.06% DW to 0.44% DW, whereas L-hArg concentrations varied from 0.16% DW to 0.90% DW. Concentrations of β -ODAP and L-hArg showed a positive correlation ($r = 0.40$, $p < 0.01$). Glutamic acid was identified as the most abundant free amino acid, followed by arginine, aspartic acid, and alanine. Genotypes with mean β -ODAP concentrations below 0.15% DW were identified as suitable for cultivation and food processing. β -ODAP and L-hArg were significantly influenced by the environment, whereas the genotype significantly affected L-hArg concentration. The location with the lowest amount of rainfall during the flowering period exhibited the highest β -ODAP concentrations. For the first time, we identified genotypes and environmental conditions with positive health-promoting and low neurotoxin attributes under Central European conditions.

1 | Introduction

Climate change affects agricultural production in temperate climates through periods of droughts, and an increased frequency of heavy rainfall events (Brasseur et al. 2023; Geilfus et al. 2024). As a result, yield instability of crops is increasing (Reckling et al. 2018a). Yield fluctuations are considered higher for spring-sown grain legumes than for autumn-sown cereals

(Reckling et al. 2018b). At the same time, the demand for plant-based protein sources is rising in Europe (van Loon et al. 2023). Therefore, there is the need for alternative legumes that are well adapted to changing climatic conditions. Grass pea (*Lathyrus sativus* L.) has great potential as a climate-resilient crop in temperate climates due to its high tolerance to both drought and waterlogging (Lambein et al. 2019; Gon  alves et al. 2022). Its high protein content and amount of polyunsaturated fatty acids

Abbreviations: L-hArg, L-Homoarginine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; DW, dry weight; β -ODAP, β -N-oxalyl-L- α , β -diaminopropionic acid.

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give grass pea great potential to serve as a valuable food grain legume (Grela et al. 2010; Ramya et al. 2022), which could also play an important role in mainstreaming diversification crops through institutional catering (André et al. 2024). Despite this potential, grass pea cultivation for human consumption is currently limited, primarily to some countries in Central and West Asia, East Africa, and in the Mediterranean region (Vaz Patto and Rubiales 2009; Kumar et al. 2011). In these regions, grass pea is regarded as an “insurance crop” for smallholder farmers, as it provides relatively stable yields under adverse environmental conditions while being cultivated on marginal lands with low yields. However, it has been shown that grass pea has higher yield potential in suitable environments (Tsegaye et al. 2005; Rath et al. 2021).

Grass pea has been largely overlooked in plant breeding programs. The main reason for this neglect is the presence of the neurotoxin β -N-oxalyl-L- α , β -diaminopropionic acid (β -ODAP) (Vaz Patto and Rubiales 2014), which is the causing agent of lathyrism, a disease characterized by irreversible spastic paralysis of the legs (Kumar et al. 2011; Lambein et al. 2019). Symptoms occur when grass pea is consumed as a staple food for at least three months in an unbalanced diet (Kumar et al. 2011). The absence of a threshold level of β -ODAP that poses no risk to human health remains an obstacle to the broader use of grass pea. However, genotypes with β -ODAP levels below $<0.15\%$ were described as low-toxin varieties and are expected to be safe for human consumption (Sacristán et al. 2015). Grass pea contains two ODAP isomers (α and β), with the β -isomer being the dominant and more toxic molecule, comprising about 95% of total ODAP concentration (Bell and O'Donovan 1966; Harrison et al. 1977).

Food processing methods, such as presoaking in water followed by discarding the soaking water and subsequent cooking, can convert up to 40% of β -ODAP into its less toxic isomer α -ODAP (Padmajaprasad et al. 1997). Buta et al. (2020) reported that pressure cooking after discarding the soaking water reduced ODAP concentrations by 16%–27%. Even greater reductions were observed after fermentation, with up to 62%–97% (Yigzaw et al. 2004; Buta et al. 2020). However, complete removal is not possible and no β -ODAP-free genotypes have currently been identified in grass pea. The factors driving the concentration of β -ODAP are related to genetics, but there are indications that environmental factors are also relevant. Little knowledge about the magnitude of the genotype-by-environment interaction makes breeding efforts for reduced β -ODAP content particularly challenging (Haque et al. 2011; Arslan et al. 2017; Aloui et al. 2023).

The role of β -ODAP in crop physiology remains poorly understood, although high β -ODAP levels have been associated with favorable traits like early flowering, resistance to insects, and higher yields (El-Moneim et al. 2000; Das et al. 2021). Additionally, β -ODAP concentrations tend to increase under environmental stress, suggesting a potential role in grass pea's tolerance to drought and flooding (Jiao et al. 2011; Verma et al. 2022; Aloui et al. 2023). Grass pea contains reasonable concentrations of L-homoarginine (L-hArg), which, unlike the undesired β -ODAP, has been linked to cardiovascular health benefits (Bell 1962; Rao 2011; Singh and Rao 2013). The positive effects on cardiovascular health result from L-hArg being

involved in nitric oxide formation, both as a substrate of nitric oxide synthase and through the inhibition of arginase (Hrabák et al. 1994; Sacristán et al. 2015). This dual action enhances nitric oxide availability, which plays a key role in vasodilation, blood pressure regulation, and overall endothelial function; mechanisms that are essential for maintaining cardiovascular health. L-Homoarginine also serves as a precursor for lysine (Stevens and Bush 1950). Concentrations of L-hArg are higher than those of β -ODAP in grass pea (Fikre et al. 2008; Onar et al. 2014). The L-hArg and β -ODAP concentrations show positive correlations; thus, crops with lower β -ODAP concentrations have lower L-hArg concentrations (Piergiorganni and Damascelli 2011; Onar et al. 2014; Sacristán et al. 2015). Research has focused on reducing β -ODAP, and the potential positive impacts of L-hArg and the role of free amino acids have received little attention.

To identify avenues for reducing the β -ODAP concentrations, we established a multienvironment study and tested 96 diverse grass pea genotypes. The genotypes were tested at three locations in Germany in 2023 and analyzed for the α -, β -ODAP, L-hArg, and for 17 other free amino acid concentrations. The objectives of the study were (i) to determine the concentration of the nonproteinogenic amino acids β -ODAP and L-hArg and 17 other free amino acids of contrasting grass pea genotypes; (ii) to assess the stability of β -ODAP and L-hArg concentrations in grass pea genotypes across different locations; (iii) to define the correlation pattern between β -ODAP and L-hArg; and furthermore (iv) to describe associations of β -ODAP and L-hArg concentrations with morphological and yield-related traits of grass pea.

2 | Materials and Methods

2.1 | Plant Material

The plant material used in the present study consisted of a set of 96 grass pea (*L. sativus* L.) genotypes from the German Genebank at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben. Four control cultivars that were used by farmers during our study were included, resulting in a total of 100 genotypes tested at each location (list in Table S1). The genotypes originated in total from 23 countries, with Italy being the most dominant origin, followed by Albania and Slovakia. The countries of origin were clustered into six groups of origin, containing countries with the most similar climatic conditions and geographical proximity (Table 1). Eight genotypes had no information about the origin and one genotype originated from Peru; these nine genotypes were therefore not assigned to any group.

2.2 | Field Trials and Experimental Design

The field trials were conducted in the summer season 2023 at three locations in Germany: the organic research station Kleinhohenheim (UHOH, 48°44'08.8"N 9°12'11.8"E) of the University of Hohenheim, the Research Centre Dedelow of the Leibniz Centre for Agricultural Landscape Research (ZALF, 53°36'99"N 13°80'13"E), and the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben (IPK, 51°49'43.7"N 11°16'26.9"E). The locations differ in climatic

TABLE 1 | Origin groups and corresponding countries of origin of the grass pea genotypes tested in the study.

Origin group	Countries of origin
Central Europe	Czech Republic (2), France (1), Germany (2), Hungary (2), Slovakia (9)
Eastern Mediterranean	Albania (10), Bulgaria (1), Greece (6), Israel (1), Turkey (3)
Western Mediterranean	Italy (30), Spain (6), Tunisia (2)
East Africa	Ethiopia (2)
West Asia	Azerbaijan (1), Iran (3), Iraq (1), Russia (2), Ukraine (3)
South Asia	India (2), Afghanistan (1), Tajikistan (1)

Numbers in parentheses indicate the number of genotypes originating from each country.

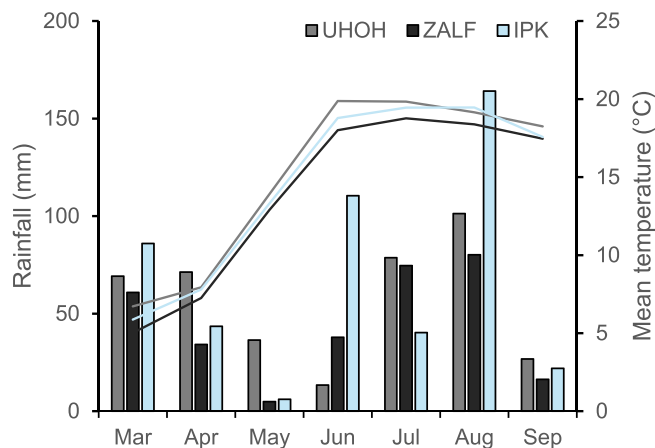


FIGURE 1 | Monthly amount of rainfall (mm) and mean temperatures (°C) from March 2023 to September 2023 at UHOH, ZALF, and IPK (data: Agrarmeteorologie Baden-Württemberg, 2023; WetterKontor, 2023; weather station Gatersleben, 2023).

conditions (Figure 1), with the lowest amount of rainfall during flowering observed at UHOH (13 mm), followed by ZALF (38 mm), and IPK (111 mm). Soil sampling was done before sowing to obtain the mineral nitrogen content ($N_{\min}$) of the locations. The $N_{\min}$ varied between 39 and 50 kg ha⁻¹ in all three locations in the upper 30 cm of topsoil.

Each genotype was grown at plots that consisted of two plant rows with 25 seeds sown in each row, resulting in 50 seeds per genotype. Sowing dates were on the 5th of April (IPK), 13th of April (ZALF), and 4th of May (UHOH). Plots were hand-sown at UHOH; at IPK and ZALF, the sowing was done mechanically with a trial plot drill (Hege 80 at ZALF, HALDRUP SP 25 at IPK). Seed to seed distance was 4 to 5 cm. The distance between the plant rows was 12 to 30 cm. Within the plant rows, trellises were installed to support plant growth, avoid lodging, and facilitate the assessments. Plots were kept weed-free by regular hand-weeding. Harvest of the plots was done by hand, and the plants were threshed afterwards. The harvest period was from 13th July till 23rd August.

The field trials were arranged as an alpha-lattice design (Patterson and Williams 1976) with 10 incomplete blocks containing 10 genotypes. The genotypes were unreplicated at each location, and the locations were regarded therefore as replicates. Field trial design was performed using the software CycDesign (VSNi 2023).

2.3 | Field Measurements and Observations

The grass pea genotypes were evaluated based on a reduced set of descriptors for *Lathyrus* spp. illustrated by IPGRI (2000), assessing both quantitative and qualitative traits. In total, 28 traits were assessed. Nine morphological and yield-related traits were included in the correlation analysis with β -ODAP and L-hArg concentrations. Plant height was measured on five randomly selected plants during flowering. Seed area and thousand-seed weight were determined using an image-based method with a seed analyzer (MARViTECH, Marvin, Version 6.0). Seeds per pod were counted from 10 randomly collected pods at harvest, and pod length and width were measured from the same sample. The number of pods per plant was assessed on 10 plants at harvest. Seed yield was expressed as grams per plant (g plant⁻¹) due to variation in the number of plants per plot. Seeds were dried to constant weight at 40°C and used as the basis for dry weight-related calculations. Seed color was classified into five categories: white, white with brown seam, white with brown seam and brown mottling, gray mottling, and gray-brown marbling. The color of the flower standard petal and wings was recorded in four categories (white, white-blue, blue, pink).

2.4 | Specific Metabolite Analysis

The concentration of seed α -, β -ODAP, L-hArg, and free amino acids was analyzed as described by Chen et al. (2000) but using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method instead of high-performance liquid chromatography with UV detection (HPLC–UV). Due to the limited seed amount of harvested grass pea genotypes and a pretesting of 10 genotypes for the establishment of the method, 90 grass pea genotypes could be analyzed. Seed samples were ground in a cyclone mill (Retsch ZM200, Germany). Twenty milligrams of each sample that had been ground to a powder were extracted using a volume of 1 mL of 80% methanol/20% water (v/v). The samples were subsequently extracted at room temperature in a Thermomixer for 1 h. The samples were then transferred to an ultrasonic bath (Elmasonic, Germany) for 5 min and then centrifuged afterwards at 20,000 g (Eppendorf, Germany). The supernatant was dried down in XcelVap (Horizon Technology Inc., Salem, NH, USA) and subsequently dissolved in 350 μ L borate buffer. A total of 70 μ L of the dissolved sample was derivatized by the addition of 10 μ L water and 20 μ L of 10 μ g/ μ L AQC tag solution (Cayman Chemical Company, Ann Arbor, MA, USA). Calibration curves were constructed using standard substances of β -ODAP, amino acid mixture AAS18 (Sigma-Aldrich, St. Louis, MO, USA), and L-homoarginine (Biomol, Hamburg, Germany). The calibration curve for β -ODAP was also employed for the quantification of α -ODAP. Prior to LC–MS/MS analysis, AQC-derivatized

samples and standards were diluted in water at a ratio of 1:100 (v/v).

LC-MS/MS analysis was performed using an Agilent 1290 II UHPLC (Agilent, Waldbronn, Germany) coupled to an AB Sciex 6500+ QTRAP MS (Framingham, MA, USA) equipped with a Turbo V ion source. Chromatography separation of AQC-derivatized amino acids was achieved on a ZORBAX Eclipse C18 column (1.8 μ m, 2.1 $\times$ 5 cm (Agilent, Waldbronn, Germany) maintained at 40°C. Gradient elution was performed using water, 0.2% formic acid (v/v) (solvent A) and acetonitrile, and 0.2% formic acid (v/v) (solvent B) as mobile phases at a flow rate of 0.3 mL/min. The following gradient was employed: 0.0 min = 2% B; 6.0 min = 6% B; 6.5 min = 10% B; 7.5 min = 20% B; 8.5 min = 25% B; and 10.2 min = 95% B, and 13.2 min = 95% B. In total, 2 μ L of each standard solution and sample was injected. The 6500+ QTRAP MS was operated in positive ion mode with multiple reaction monitoring (MRM) detection to quantify α -ODAP, β -ODAP, L-hArg, and other amino acids (Table S2). The conditions used for the 6500+ QTRAP mass spectrometer were as follows: electrospray voltage, +4000 V; source temperature, 450°C; nebulizer gas (gas 1), 60 psi; heater gas (gas 2), 70 psi; curtain gas, 50 psi; and collision gas (CAD), 9 psi. Air was utilized as nebulizer gas and heater gas, whereas nitrogen was used as collision gas and curtain gas. MRM transitions specific for α -, β -ODAP, and L-hArg were observed at elevated concentrations in standard solutions. These transitions were used to optimize chromatography conditions, thereby facilitating the chromatographic separation of α -, β -ODAP, and L-hArg.

2.5 | Statistical Analysis

Analysis of variance (ANOVA) was done using a mixed model, with genotype and location as fixed effects and blocks within the location as random. To account for heterogeneity between locations, location-specific block and error variances were allowed in the model. Normal distribution of residuals and homogeneity of variance were checked graphically before conducting the ANOVA. Statistical analyses were performed in SAS, using the PROC MIXED with restricted maximum likelihood (REML) procedure to estimate variances (SAS Institute Inc. 2023). The global null hypothesis of no genotype differences was tested using Wald-type *F*-tests. *F*-tests were followed by Tukey's test as a post hoc test to examine mean differences. Means for the traits, β -ODAP and L-hArg were obtained for all genotypes across locations and were used to determine Pearson's correlation coefficients. Pearson's correlation coefficients were calculated using the PROC CORR procedure in SAS (SAS Institute Inc. 2023).

TABLE 2 | Minimum, mean, maximum values, and coefficient of variation (CV) and overall mean with standard deviation ($\pm$ SD) for β -ODAP and L-hArg concentrations (% DW) at the three locations (UHOH, IPK, ZALF).

	UHOH				IPK				ZALF				Overall
%	Min	Mean	Max	CV	Min	Mean	Max	CV	Min	Mean	Max	CV	Mean $\pm$ SD
β -ODAP	0.11	0.23	0.44	25.0	0.06	0.15	0.26	29.7	0.07	0.15	0.28	27.3	0.18 $\pm$ 0.06
L-hArg	0.21	0.43	0.90	32.5	0.16	0.35	0.84	38.0	0.22	0.49	0.86	27.0	0.43 $\pm$ 0.15

Due to missing data, the number of observations per genotype and location slightly varies ($n = 251$).

3 | Results

3.1 | Concentration of α -, β -ODAP, and L-hArg

The average β -ODAP concentration across all locations was 0.18% DW ($\pm$ 0.06), with a range from 0.06% DW to 0.44% DW. Lower mean β -ODAP concentrations were observed at ZALF and IPK (both 0.15% DW), in contrast to UHOH, where the mean β -ODAP concentration was significantly higher (0.23% DW) (Table 2). The distribution of β -ODAP concentrations was similar at ZALF and IPK, indicating no significant difference between the two locations (Figure 2).

Of the 90 tested genotypes, 14 had a mean β -ODAP concentration below 0.15% across the three locations. The α - and β -ODAP concentrations were significantly influenced by the location, whereas the genotypic influence was not significant, highlighting the environmentally dependent variability of ODAP concentration among the genotypes (Table 3).

Low between-location Spearman rank correlations for β -ODAP concentration (Spearman's $\rho = 0.01$ –0.31, Table S3) indicate pronounced genotype re-ranking and instability across locations, indicating that genotype-by-environment interactions were present for β -ODAP concentration. Concentrations

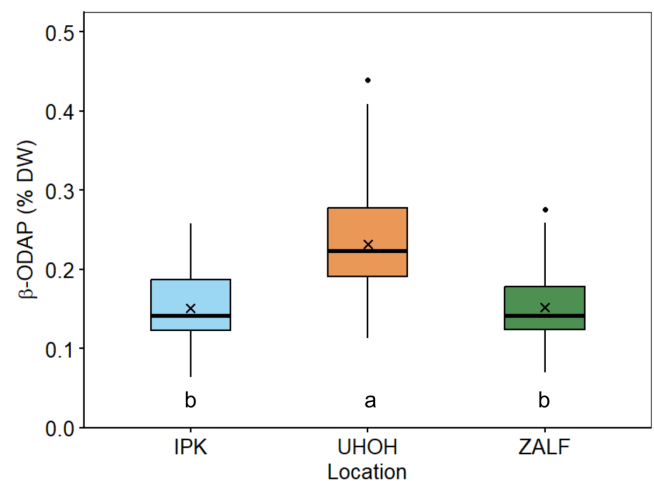


FIGURE 2 | Boxplot showing the distribution of β -ODAP concentration (% DW) across locations. The boxes represent the interquartile range, with the horizontal line indicating the median. Whiskers extend to the minimum and maximum values, whereas points outside the range are considered outliers and represented as individual dots. Different letters indicate significant differences between locations based on ANOVA with Tukey's HSD, $p < 0.01$; $n = 3$.

TABLE 3 | Results of mixed model analysis performed for α -, β -ODAP, and L-hArg concentrations (% DW) on 90 grass pea genotypes at the three locations (UHOH, IPK, ZALF), ($n = 251$).

	Source of Variation	Num DF	Den DF	F-value	Pr > F
β -ODAP	Genotype	89	7.88	1.99	0.1504
	Location	2	13.00	32.04	<0.0001
α -ODAP	Genotype	89	1.00	2.09	0.5086
	Location	2	14.10	174.00	<0.0001
L-hArg	Genotype	89	103.00	1.69	0.0052
	Location	2	13.90	21.64	<0.0001

TABLE 4 | Mean ($\pm$ standard error) β -ODAP and L-hArg concentration within the respective origin group.

Origin group	n	β -ODAP	L-hArg
Central Europe	41	0.18 $\pm$ 0.01	0.49 $\pm$ 0.02
Eastern Mediterranean	54	0.18 $\pm$ 0.01	0.36 $\pm$ 0.01
Western Mediterranean	97	0.19 $\pm$ 0.01	0.46 $\pm$ 0.01
East Africa	4	0.16 $\pm$ 0.03	0.38 $\pm$ 0.01
West Asia	22	0.17 $\pm$ 0.01	0.40 $\pm$ 0.02
South Asia	9	0.17 $\pm$ 0.02	0.37 $\pm$ 0.03
Unknown	24	0.16 $\pm$ 0.03	0.40 $\pm$ 0.01

ranged from 0.06% in LAT 438 at IPK to 0.44% in LAT 4084 at UHOH. On average, 1.9% of the total ODAP concentration accounted for α -ODAP. Mean β -ODAP concentration was highest in genotypes from the Western Mediterranean (Table 4). Seed and flower color were not related to increasing or decreasing β -ODAP concentration.

The L-hArg concentrations were in general about 2.5-fold higher than β -ODAP concentrations, showing a mean across locations of 0.43% DW (± 0.15), with a range from 0.16% DW to 0.90% DW. Highest mean L-hArg concentrations were found at ZALF (0.49% DW), followed by UHOH (0.43% DW) and IPK (0.35% DW) (Table 2; Figure 3). Likewise with the highest β -ODAP concentration found for the genotype LAT 4084 (Italian origin, Island Vewtotene, East of Naples) at location UHOH, the highest L-hArg concentration (0.90% DW) was also found for this genotype at UHOH. Unlike the ODAP concentration, L-hArg concentration was determined by the genotype ($F = 1.69$, $p < 0.01$; Table 3), but the influence of location was also significant for L-hArg concentration. Genotype rank correlations for L-hArg concentration between locations were limited (Spearman's $\rho = 0.29$ – 0.34 ; Table S4), indicating significant variation in genotype rankings for L-hArg concentrations across locations and present genotype-by-environment interactions.

L-hArg concentration showed a higher variation than β -ODAP concentrations at the respective location, except for ZALF (Table 2). Highest mean L-hArg concentration was observed with the origin group of Central Europe (Table 4). Genotypes

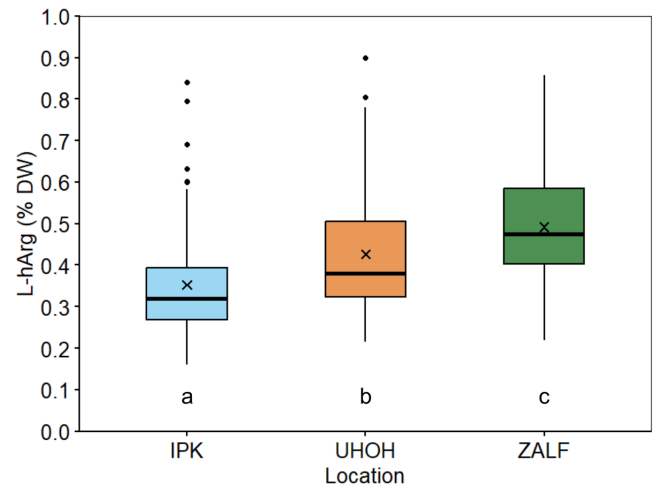


FIGURE 3 | Boxplot showing the distribution of L-hArg concentration (% DW) across locations. The boxes represent the interquartile range, with the horizontal line indicating the median. Whiskers extend to the minimum and maximum values, whereas points outside the range are considered outliers and represented as individual dots. Different letters indicate significant differences between locations based on ANOVA with Tukey's HSD, $p < 0.01$; $n = 3$.

with a white standard petal exhibited significantly higher L-hArg concentrations than those with a blue standard petal (Tukey's HSD, $p < 0.01$). Genotypes with white seeds had the highest L-hArg concentrations; however, the difference was statistically significant only when compared to seeds with gray-brown marbling (Tukey's HSD, $p < 0.01$).

3.2 | Free Amino Acid Concentrations

Across locations, the amino acid glutamic acid was the most abundant amino acid ($9.78 \text{ mg } 100 \text{ g}^{-1}$), followed by arginine ($6.40 \text{ mg } 100 \text{ g}^{-1}$), aspartic acid ($5.77 \text{ mg } 100 \text{ g}^{-1}$), alanine ($4.43 \text{ mg } 100 \text{ g}^{-1}$), and glycine ($2.48 \text{ mg } 100 \text{ g}^{-1}$) (Table 5). The lowest amount was detected for threonine and cysteine. No cysteine was found in genotypes grown at UHOH and IPK; moreover, in 12 genotypes grown at ZALF, cysteine was found in small quantities ($0.01 \text{ mg } 100 \text{ g}^{-1}$ to $0.05 \text{ mg } 100 \text{ g}^{-1}$). The mean concentration of the 17 measured free amino acids was highest at ZALF. Furthermore, the order of the highest concentrated amino acids differed at ZALF compared to IPK and UHOH. At IPK and UHOH, the second most abundant

free amino acid was aspartic acid, whereas it was arginine at ZALF. The highest variation was indicated for threonine, serine, and proline (Table 5). All amino acids were significantly positively correlated with L-hArg, with arginine showing the strongest correlation ($r=0.59$; $p<0.01$). Weak correlations with β -ODAP were observed for glutamic acid, aspartic acid, glycine, methionine, and serine. No significant negative correlations with either β -ODAP or L-hArg were detected (data not shown).

3.3 | L-hArg and β -ODAP Associate With Morphological and Yield-Related Traits

The L-hArg and β -ODAP concentrations had a positive correlation ($r=0.40$, $p<0.01$) (Table 6), which was independent of locations. The β -ODAP concentration showed a weak negative correlation to yield, whereas the L-hArg concentration was positively correlated to plant yield. Early flowering increased both β -ODAP and L-hArg concentrations; however, the correlation was weak for L-hArg. The β -ODAP concentration decreased with a higher number of pods per plant and seeds per pod but increased with pod size. Higher L-hArg concentrations corresponded to increased seed and pod size. A high number of seeds per pod decreased L-hArg levels,

TABLE 5 | Summary statistics (minimum, maximum, mean $\pm$ SD, and coefficient of variation [CV %]) of the concentrations of 16 free amino acids (mg 100 g⁻¹ DW), across 90 grass pea genotypes at three locations in Germany.

Amino acid	Min	Max	Mean $\pm$ SD	CV (%)
Glutamic acid	4.35	21.67	9.78 $\pm$ 2.75	28.12
Arginine	2.07	22.26	6.40 $\pm$ 3.12	48.75
Aspartic acid	1.18	13.19	5.77 $\pm$ 2.06	35.70
Alanine	1.51	14.98	4.43 $\pm$ 2.06	46.50
Glycine	1.22	6.11	2.48 $\pm$ 0.74	29.84
Phenylalanine	0.62	3.93	1.56 $\pm$ 0.54	34.62
Valine	0.59	3.74	1.35 $\pm$ 0.51	37.78
Proline	0.40	6.26	1.11 $\pm$ 0.65	58.56
Leucine	0.48	4.28	1.23 $\pm$ 0.53	43.09
Lysine	0.34	3.52	1.01 $\pm$ 0.53	52.48
Tyrosine	0.26	2.76	0.71 $\pm$ 0.34	47.89
Histidine	0.23	2.12	0.70 $\pm$ 0.35	50.00
Methionine	0.21	1.17	0.55 $\pm$ 0.19	34.55
Serine	0.18	2.40	0.54 $\pm$ 0.33	61.11
Isoleucine	0.15	1.60	0.47 $\pm$ 0.23	48.94
Threonine	0.15	1.63	0.39 $\pm$ 0.24	61.54

Due to missing data, the number of observations per genotype and location slightly varied ($n=251$).

whereas a greater number of pods per plant slightly increased L-hArg.

3.4 | Plant Yield

Mean plant yield was highest at location ZALF (12 g plant⁻¹), followed by UHOH (4 g plant⁻¹) and IPK (2 g plant⁻¹). The average plant yield across locations was 5.89 g plant⁻¹ (± 5.12). A mixed model analysis revealed a significant effect of location on yield ($F=112.22$, $p<0.01$; Table 7). The genotypic effect was not significant, suggesting that genotypes exhibited similar yields within each location. At all three locations, genotypes with higher yields than the control cultivars were identified. Two genotypes (LAT 4008 and LAT 4010) had below 4 g plot yield at IPK and were excluded from yield analysis. Therefore, the dataset comprised 298 observations instead of the full 300. The highest yielding genotypes at each location were LAT 454 (24 g plant⁻¹, ZALF), LAT 413 (6 g plant⁻¹, UHOH), and LAT 4031 (4 g plant⁻¹, IPK).

TABLE 6 | Pearson correlation coefficients and associated p -values between β -ODAP, L-hArg, and 9 traits (PH=plant height, SA=seed area, NSP=number of seeds per pods, PodL=length of pod, PodW=width of pods, NPP=number of pods per plant, FL=days to flowering, TSW=thousand-seed weight, SY=seed yield plant⁻¹) based on genotypic means. Asterisks represent the significance of correlations (* $p<0.05$, ** $p<0.01$), significant correlations are highlighted in bold.

	β -ODAP	L-hArg
L-hArg	0.40**	
PH	0.13*	-0.14*
SA	-0.06	0.37**
NSP	-0.20**	-0.31**
PodL	0.13*	0.40**
PodW	0.19*	0.46**
NPP	-0.39**	0.20**
FL	-0.49**	-0.16**
TSW	-0.05	0.31**
SY	-0.13*	0.31**

TABLE 7 | Results of mixed model analysis performed for plant yield (g plant⁻¹) on 100 grass pea genotypes at the three locations (UHOH, ZALF, IPK) ($n=298$).

Source of variation	Num DF	Den DF	F -value	Pr > F
Genotype	99	1	1.13	0.6510
Location	2	11	112.22	<0.0001

4 | Discussion

4.1 | Low Concentration of Nonproteinogenic Amino Acids ODAP and L-hArg

The metabolite analysis revealed that the grass pea genotypes exhibited generally low mean β -ODAP concentrations under the tested Central European conditions in Germany (Table 2). Fourteen genotypes showed β -ODAP concentrations below 0.15% and, except for two, also displayed above-average mean yields, highlighting the presence of promising candidates for further testing and cultivation in Central Europe. The highest β -ODAP concentration (0.44%) was lower than values reported in previous screenings (Onar et al. 2014; Arslan et al. 2017) and was confined to a single location and one genotype. However, no genotype completely free of β -ODAP was identified. Moderate variation in β -ODAP concentrations among genotypes within locations was observed, consistent with earlier studies reporting substantial variability within and among *Lathyrus* species (Fikre et al. 2008; Basaran et al. 2013; Onar et al. 2014; Arslan 2017; Arslan et al. 2017; Yazici et al. 2020; Abdallah et al. 2024). The range of β -ODAP concentrations observed was comparable to reported ranges from a study in Turkey (Basaran et al. 2013), indicating that environmental conditions in Central Europe do not substantially alter β -ODAP accumulation.

Unlike the commonly used spectrophotometric method, which quantifies only total ODAP (Rao 1978), the analytical approach applied here enabled differentiation between the two ODAP isomers present in grass pea. The α -ODAP to β -ODAP ratio was lower than previously reported (Bell and O'Donovan 1966), suggesting that α -ODAP contributes negligibly to total ODAP concentration. Thus, for further genotype screenings, the faster and more cost-effective method of Rao (1978) may represent an adequate analytical method.

Our data confirm that L-hArg is the most abundant free amino acid in grass pea. However, the concentrations observed were lower than those reported in previous studies (Piergiorganni and Damascelli 2011; Onar et al. 2014; Sacristán et al. 2015),

4.2 | Variation in Free Amino Acid Composition

In tested grass peas, the most abundant free proteinogenic amino acids were glutamic acid and arginine, irrespective of location, confirming earlier reports (Fikre et al. 2008). In contrast, the concentration of free lysine was low (0.34–3.52 mg 100⁻¹ DW). However, due to the lack of data on protein-bound amino acids, a comprehensive interpretation of overall lysine content is limited. The general pattern of free amino acids in grass pea was similar to those reported for other legumes such as beans, lentils, and peas (Kuo et al. 2004). A broad variation in free amino acid concentrations among grass pea genotypes has previously been described (Megías et al. 2015; Arslan et al. 2022). The low concentration of sulfur-containing amino acids in grass pea (Hanbury et al. 2000; Yan et al. 2006) was also confirmed in the tested grass pea genotypes. Free cysteine was only detectable in a few genotypes and in small

amounts at a single location. In the present study, significant variation was observed for three free amino acids, with the highest fluctuations in threonine. This disagrees with results from Arslan et al. (2017), who identified arginine as the most variable free amino acid among grass pea genotypes, suggesting that growing conditions in Central Europe may influence the relative variability of amino acids. Arginine is a precursor in the biosynthesis of L-hArg (Koch et al. 2023) and a positive correlation between the two compounds was confirmed in this study. It was shown that a reduction in β -ODAP concentration did not result in an increase of other amino acids, indicating that no compensatory accumulation occurs when β -ODAP biosynthesis is reduced. The genotypic differentiation of free amino acid concentrations may serve as a useful criterion for the selection of stress-resilient germplasm. In particular, proline is well known for its involvement in drought stress responses (Lehr et al. 2022) and has been associated with improved drought tolerance in grass pea (Arslan 2017; Tokarz et al. 2020). However, in the current study, no significant genotypic or location differences in proline concentration were observed.

4.3 | Environmental Effects on β -ODAP and L-hArg Accumulation

Environmental and genetic variability of both β -ODAP and L-hArg, has been reported in various studies (Fikre et al. 2011; Piergiorganni and Damascelli 2011; Girma and Korbu 2012; Arslan et al. 2017; Tadesse 2003). Although there is evidence of genetic determination for β -ODAP, environmental conditions can significantly influence its concentration, particularly under stress factors such as drought. Tadesse (2003) and Jiao et al. (2011) highlighted the impact of differing environmental conditions on β -ODAP concentrations. In our field trials, differences in β -ODAP concentration were not observed in the genotypes; however, significant differences were present for the locations (Table 2). This contrasts with the results from Hanbury et al. (1999), which exhibited a greater impact of genotype than the environment on β -ODAP concentrations. Grela et al. (2010) found that β -ODAP concentration decreases with a higher concentration of tannin. Results from the present study can however not support these findings. The presence of genotype-by-environment interaction, as shown by the change in genotype performance ranking across environments, indicates that genotype selection for low β -ODAP concentration must consider specific environments. Yan et al. (2006) reported that genotypes with low β -ODAP concentrations were not stable across locations. Similar trends were observed under the Central European conditions tested here. The β -ODAP concentration is known to increase under drought and heat stress (Xiong et al. 2014; Aloui et al. 2023). Therefore, the lower amount of rainfall during the flowering period at location UHOH points to increased β -ODAP concentrations. A study in Italy from Piergiorganni et al. (2010) revealed similar results by increased β -ODAP concentrations under low rainfall conditions. The enhanced accumulation of secondary metabolites, including nonproteinogenic amino acids, under stress conditions through metabolic reprogramming is well documented (Upadhyay et al. 2025) and may

explain the higher β -ODAP concentrations observed under drought conditions in this study. Although within-location variation was comparable at IPK and ZALF, it was greater at UHOH. This suggests that the location effect itself was less pronounced than the influence of reduced rainfall during flowering, showing different genotypic responses to the stress conditions.

Concentrations of L-hArg were influenced by both genetic and environmental factors. Genotypic differences were evident, as significant effects of genotype origin as well as seed and flower color on L-hArg concentration were observed. The highest L-hArg concentrations were present at the highest yielding location. Fluctuations among environments could therefore result from simultaneous increases of yield alongside L-hArg levels.

4.4 | Pattern of β -ODAP and L-hArg

A positive correlation between β -ODAP and L-hArg concentrations was observed ($r=0.40$; Table 6). Although the correlation was weak, it was statistically significant, indicating that reduced β -ODAP concentrations are also associated with reduced L-hArg concentrations. Similar associations have been reported previously (Piergiorganni and Damascelli 2011; Onar et al. 2014; Sacristán et al. 2015). Our findings further support this pattern, as the highest concentrations of both β -ODAP and L-hArg were detected in the same genotype, similar to the results reported by Onar et al. (2014).

4.5 | Impact of β -ODAP and L-hArg Concentration on Morphological and Agronomic Performance

In the tested grass pea genotypes, β -ODAP concentrations were higher in early flowering genotypes (Table 6), consistent with findings from El-Moneim et al. (2000). For homogeneous ripening, especially under Central European conditions, early flowering genotypes are generally preferred, indicating a problem in the selection of suitable genotypes. A slight negative correlation was observed between plant yield and β -ODAP concentration, as well as between β -ODAP and the number of pods per plant. Therefore, higher yields lead to lower β -ODAP concentration. This disagrees with El-Moneim et al. (2000) and Basaran et al. (2013) stating that low β -ODAP genotypes have lower seed yield. However, a dilution effect with the accumulation of higher biomass might partially explain the reduced β -ODAP concentration observed in high-yielding genotypes. This interpretation is further supported by a negative correlation between β -ODAP concentration and thousand-seed weight reported in other studies (Dutta et al. 2024). Such a potential dilution effect might also reduce the impact of environmental factors on β -ODAP concentrations. In contrast, L-hArg concentrations were found to increase in association with seed yield and yield-related traits such as the number of pods per plant, thousand-seed weight, and seed size, indicating that high-yielding genotypes accumulate more L-hArg or that favorable environments increase both yield and L-hArg concentrations.

5 | Conclusion

Grass pea represents a valuable alternative grain legume crop with promising potential under changing growing conditions. Our results demonstrate that it can be successfully cultivated with low β -ODAP concentrations under Central European environments such as those in Germany. In addition, the presence of L-hArg enhances its nutritional value. The identified genotypes may therefore serve as valuable parent germplasm for future breeding processes.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Summary of tested genotypes with accession name, cultivar/trade name, country of origin (ISO 3166-1 country codes), accession URL from gene bank at IPK, and PUID (persistent unique identifier). **Table S2:** Parameters used for MRM of target compounds. The table lists the compound identifiers and the optimized instrument settings for each MRM transition, including retention time, retention time tolerance, precursor ion (Q1 mass), product ion (Q3 mass), and instrument parameters: declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). **Table S3:** Spearman's rank correlation coefficients (ρ) for β -ODAP concentration (% DW) among the three locations. Genotypes with the lowest β -ODAP concentrations at the respective locations are described with the best ranks. **Table S4:** Spearman's rank correlation coefficients (ρ) for L-hArg concentration (% DW) among the three locations. Genotypes with the highest L-hArg concentrations at the respective locations are described with the best ranks.