Intraspecific variability overlays abiotic site effects on some quality parameters of walnut (*Juglans regia* L.) fruits from Kyrgyzstan

Sabrina Alica Meisen¹ · Jamila Smanalieva² · Zhyldyz Oskonbaeva^{1,3} · Janyl Iskakova³ · Dietrich Darr¹ · Florian Wichern¹

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Abstract

Kyrgyz walnut-fruit forests harbour a unique walnut diversity, which has rarely been investigated concerning nut properties and the influence of environmental conditions on these. We evaluated the influence of soil properties and altitude on physical and some chemical walnut properties at three sampling sites differing in altitude by 200 m. Walnut samples were collected from 15 randomly chosen trees. Soil samples from two depths under each tree were analysed for plant available mineral nutrients and soil chemical properties. In contrast to our hypothesis, physical nut and chemical kernel quality parameters did not differ between sampling sites at different altitude and were not affected by soil properties. Only pH showed a relationship with manganese availability in soil and kernel content, and was related to amino acid content of kernels. Tree and site-specific variability overrides abiotic influence caused by different altitude or soil properties and reflect the high genetic variability in these forests.

Keywords Abiotic factors · Mineral nutrient content · Quality · Soil properties · Walnut

Introduction

The Republic of Kyrgyzstan is home to the largest naturally occurring walnut (*Juglans regia* L.) forest areas of the world [1], which are found in the Fergana Range North and East of Jalal-Abad at an elevation between approximately 1000 and 2000 m above sea level [2]. After the last glacial period, stable climatic conditions prevailed for 4000 years

and facilitated the development of a unique ecosystem of high value for the local community, which make use of the various biological resources [1]. Next to walnuts, the forests also harbour wild fruit trees of which some are endemic and show high potential as a health benefitting food source due to valuable secondary plant metabolites [3]. Even though cross-pollination and thus genetic drift is favoured by the monoecious and dichogamous reproduction system of *J. regia* [4], relatively low allelic variability was found amongst Kyrgyz walnuts, reflecting a genetically unique biological resource [5, 6], which may harbour special walnut quality traits and intraspecific variability. Knowledge about this, however, is lacking.

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✉ Florian Wichern
florian.wichern@hsrw.eu

Sabrina Alica Meisen
Sabrina-Alica.Meisen@hsrw.org

Jamila Smanalieva
jamila.smanalieva@gmail.com

Zhyldyz Oskonbaeva
zhyldyz.oskonbaeva@gmail.com

Janyl Iskakova
janil.iskakova@gmail.com

Dietrich Darr
Dietrich.Darr@hochschule-rhein-waal.de

¹ Faculty of Life Sciences, Rhine-Waal University of Applied Sciences, Germany Marie-Curie-Str. 1, 47533 Kleve, Germany

² Department of Food, Nutrition and Home Economics/Vocational Didactics, Institute of Vocational Education and Vocational Didactics, Technical University Dresden, Weberplatz 5, 01217 Dresden, Germany

³ Engineering Faculty, Environmental Engineering Department, Kyrgyz-Turkish Manas University, pr. Aytmatov 54, 720044 Bishkek, Kyrgyzstan

The walnut fruit forests are inhabited by around 40,000 people, which are highly dependent on the resources provided by the ecosystem to sustain their subsistence. Parts of the forest are leased to private households and used for wild fruit, fuelwood and hay collection as well as grazing and horticultural activities, which result in overexploitation of the biological resources with reduced forest rejuvenation and increased erosion [1]. Local people collect walnuts in autumn and sell these on local markets from where the nuts are partly exported to neighbouring countries. As a significant proportion of walnut use and trade is informal, it is not reflected in Kyrgyzstan's official walnut production and trade statistics; World total walnut production amounted to 3,829,626 metric tons in 2017 with China that contributes a share of 50% being the largest producer. Other major walnut producers are the USA, Iran, Turkey, Mexico, Ukraine, Chile, Uzbekistan, Romania and France [7]. Nonetheless, the unique walnut forests may hold the potential for quality walnut production a marketing beyond the local markets.

Walnut kernels are rich in mineral elements, such as K, Mg, Ca, P and Fe but reveal quite some variability [8], probably partially related to genotypic variability [9–11] and nutrient availability in the soil [8]. Moreover, walnut kernels have high concentrations of fatty acids with nutritional value for humans [12]. On average, they are composed of 14% protein and contain all essential and semi-essential amino acids [13] and thus, provide a valuable source of protein and minerals for human nutrition [12]. In addition, they are sometimes claimed to be beneficial for human health, due to their content of secondary plant metabolites with e.g. antioxidant capacity [12], even though contents vary between genotypes [14]. However, recently, it has been shown that seasonal and environmental factors, such as temperature can affect walnut kernel quality as well [10, 15]. Fuentealba et al. [15] designated differences in metabolic compounds of walnut kernels to climatic conditions provoked by mountainous and coastal growing conditions in Chile. Higher antioxidant capacity along with higher total phenolic compounds (TPC) was found in walnut kernel samples from areas in the Andes with larger temperature variability as compared to the coastal investigation area. These differences corresponded to lighter walnut kernel colour, a visual indicator of product quality and most likely show the result of higher antioxidant capacity of walnut kernels affected by temperature, which reduces the oxidation of phenolics and thus browning reactions, leaving a higher amount of TPC and lighter kernels. Lighter coloured walnuts originating from higher altitude was also observed elsewhere [16]. Next to differences in temperature also precipitation and other site factors can affect the antioxidant capacity of walnuts [17]. Furthermore, physical nut parameters, such as shell hardness changed with altitude and latitude due to altered light and temperature conditions [5, 16, 18]. Also, water

availability has been observed to affect physical walnut and kernel properties [19], even though genetic variability causes large differences as well [6], leaving the question if genotype or environmental factors stronger influence walnut quality unanswered [10].

In the light of these results, it is important to consider future climate change effects, which will result in increasing average temperatures and more pronounced drought periods in many parts of Central Asia [20], as they will most likely also affect the quality of walnuts from the walnut fruit forests. To assess the potential temperature effect, sites that vary with regard to elevation and thus average temperature may be used as a proxy for future temperature scenarios. In this context, our research aimed at analysing physical and some biochemical walnut quality parameters, which have rarely been investigated in Kyrgyz walnuts before. As genetic variability is expected to be comparable at all sites, we assume that differences in average temperature caused by different altitude have a pronounced effect on walnut quality parameter and that soil properties at the three sites affect walnut kernel properties. We hypothesized, that elevation differences affect physical and chemical walnut kernel properties, in particular total phenolic compounds, and that mineral walnut kernel composition is reflected by soil extractable mineral elements.

Materials and methods

Sampling and storage

Walnut (*Juglans regia* L.) samples (walnuts without husk) were taken in September 2017 during harvesting time at different altitudes in the natural walnut forests in Dashman (DA) and Kyzyl-Unkur (KU), located between 41°35′–41°47′N and 73°01′–73°10′E on 1258 (KU1—low), 1421 (KU2—medium), and 1685 (DA—high) meters above sea level. The differences in altitude of about 200 m between each location translates into an average temperature difference of approximately 1.3 °C. Average temperatures at KU1, KU2 and DA were 11, 10 and 8 °C respectively in 2017 as determined by data logger. Precipitation data for the region are not existent and only available from models, which can not be used to differentiate between the investigation sites. The 30 year average precipitation is approximately 825 mm per year. The true precipitation at the sites thus remains unknown, however, local people claim there be no difference between the sites. At every sampling site, 5 trees were randomly chosen and 50 fallen walnut fruit samples without husk were collected from every tree, except for tree 3 at site KU1 (KU1.3) where only 24 samples were found. Nut samples were air-dried and stored at 7 °C until analysis. From the 50 walnut fruit samples, 25 were chosen randomly

and each investigated for physical properties. After physical analysis, kernels were separated, frozen at -30°C , chopped into small pieces, vacuum packed and kept in the dark at -18°C until chemical investigation. Soil samples were taken from a depth of 0–30 and 30–60 cm in fivefold replicates under each of the trees and mixed to one composite sample per depth per tree. Fresh soil was stored at 4°C . Soil samples were sieved ($<2\text{ mm}$) and air-dried prior to analysis. All soil and walnut kernel extracts and digests analysed in this study were frozen at -4°C until measurement.

Soil properties

Soil pH and electrical conductivity (EC) were assessed with a SevenMulti device (Mettler Toledo, Gießen, Germany) using air-dried, sieved ($<2\text{ mm}$) soil and de-ionized water (1:2 w/w). Soil organic matter (SOM) was measured by loss on ignition according to Hoogsteen et al. [21]. Carbonate content measurement was conducted with a calcimeter according to the operating instructions issued by the manufacturer. Plant available macronutrients Ca, K, Mg, P and micronutrients Cu, Fe, Mn and Zn were extracted in Machigin solution according to Sakbaeva et al. [22] with modifications. Briefly, 10 g air-dried soil was suspended in 20 mL 1% NH_4CO_3 solution and shaken at 200 rpm (KS 4000 i control, IKA—Werke GmbH & Co. KG, Staufen, Germany) for 30 min. After filtering samples were analysed by ICP—OES (Optima 8000, PerkinElmer, Waltham, USA).

Walnut properties

Walnut length, width and thickness were determined with a vernier caliper and sphericity and geometric mean diameter (D_g) were calculated according to Altuntas and Erkol [19]. Fresh walnut and kernel mass were weighed with a digital electronic 4-figure balance with a sensitivity of 0.01 mg, and the kernel mass proportion (KMP in %) was calculated. Shell integrity and walnut shape were evaluated according to the IPGRI [23]. Shell hardness was determined by rupture force, which was measured along the suture line by a texture measuring instrument (Zwicki-Line Z1.0 TS, Zwick Roell Group, Ulm, Germany). After removal of the shell, the proportion of damaged and intact kernels was calculated. Damages include fully dried and partially dried kernels and those partially damaged by insects. Intact and partially intact kernels were grouped into colour categories “extra light”, “light”, “light amber”, and “amber” according to the Chilean Walnut Commission [24].

Dry matter and ash content of kernels were determined according to Gharibzadeh et al. [25]. Unless specified, for all subsequent analyses dried composite kernel aliquots were taken. A subsample of ground walnut kernel was analysed for total nitrogen content using elemental analysis (Elemental Analyzer NA1500, Thermo Scientific, Waltham,

MA, USA). Total protein content was determined by converting nitrogen values with a conversion factor of 5.3. Total fat was determined by applying the Soxhlet extraction method [26]. Unfortunately, fatty acid determination failed and could not be repeated due to a lack of sample material. Carbohydrate content was estimated according to Gharibzadeh et al. [25] using the following formula:

$$\text{Carbohydrate content} = 100\% - (\% \text{ moisture} + \% \text{ protein} + \% \text{ fat} + \% \text{ ash}).$$

The mineral elements Ca, K, Mg, P, S, Cu, Fe, Mn and Zn in walnut kernels were extracted as described by Jones [27] with modifications. Briefly, 0.5 g of kernel sample was weighed into a digestion tube and 5 mL HNO_3 (65%) was added for overnight incubation. Thereafter, samples were digested at 125°C for 1 h. Then, 9 mL H_2O_2 (30%) was added in three steps into the digestion tube and boiled at 125°C for 30 min until the digest was clear. The digest was diluted with distilled water, filtered and final volume was brought to 50 mL. Analysis was carried out with ICP—OES.

Extraction of total phenolic compounds (TPC) was carried out according to Arranz et al. [28]. TPC were determined with Folin—Ciocalteu reagent using a UV—Spectrophotometer (Genesys 10S UV—VIS, Thermo Fisher Scientific, Dreieich, Germany) at 765 nm. TPC was expressed as mg gallic acid equivalents (GAE) per gram of kernel dry weight ($\text{mg GAE g}^{-1}\text{ DW}$).

Free amino acid contents were analysed by high-performance liquid chromatography (HPLC, Series 1200 Agilent Technologies, California) according to Hermosín et al. [29], with minor modifications as follows. For the isolation of amino acids, 0.2 g of defatted sample was diluted in 1.5 mL 4-aminobutyric acid, 0.7 mL 0.1 M HCl and 1.5 mL distilled water. Derivatization was performed by adding 30 μL diethyl ethoxymethylenemalonate, 1.5 mL methanol, 1 mL sample and 3.5 mL of 1 M borate buffer (pH 9) into a test tube. The tubes were incubated in an ultrasound water bath for 30 min. at room temperature. All samples were filtered through a $0.45\text{ }\mu\text{m}$ nylon membrane prior to HPLC injection. Pure acetonitrile and 25 mM acetate buffer (pH 5.8) were used as mobile phase with a regulation of 6% acetonitrile followed by 16% (elapsed time: 13 min), 18% (13.5 min), 18% (17 min), 22% (20 min), 32% (32 min) and 32% (35 min) at a flow rate of 1 mL/min. Absorption was measured by a Diode Array Detector (DAD detection).

Statistical analyses

The open source program R 3.5.1 combined with R studio 1.1.456 was used for the statistical evaluation of the

generated data. Three statistical approaches were carried out:

1. Site-specific effects were evaluated by using five replicates per sampling site. An exception of this is count data i.e. kernel colour and condition, walnut shape, and shell integrity. It is expressed as percentage of the whole walnut fruit sampling amount per site to avoid representing an average value of only one tree.
2. After finding no significant differences between sites for most walnut and kernel properties, a tree-specific analysis was conducted independently of site origin. Based on the assumption that abiotic conditions are influencing physical walnut quality parameters similarly at all sites, effects of intraspecific variability were evaluated by using 25 kernel replicates per tree (24 kernels for KU1.3).
3. Furthermore, walnuts of all trees (374 replicates) were analysed independently of site or tree to evaluate general relationship of walnut and kernel properties (colour, shell integrity, TPC, moisture content, amino acids, mineral nutrient content) as well as their relation to soil properties (pH, extractable mineral nutrients).

Normal distribution and variance homogeneity of the data were evaluated visually by means of a Normal Q-Q plot and Residuals vs Fitted Values plot, respectively. Kruskal–Wallis tests were conducted as data were found to be non-parametric after Boxcox transformation using the package MASS. Dunn's Test was performed as post hoc test for differentiation with the FSA package in case significant differences

were found. Spearman's Rank correlation coefficient of the package agricolae was used to determine correlation coefficients for soil pH and free amino acid concentration, where classification was done according to Rumsey [30]. Kendall's τ correlation coefficient was used for evaluating the degree of dependence between plant available nutrients in the soil and mineral elements in walnut fruits. Again, Normal Q-Q and Residuals vs Fitted Values plots were utilized to examine normal distribution and variance homogeneity. A Fisher's Exact test was done to correlate shell integrity and kernel colour. A correlation test between the proportion of dried kernels and the concentration of free amino acids was done by means of logistic regression. Furthermore, ggplot2 was used to visualise data.

Results

Soil properties

Soil pH was significantly lower at site DA (highest elevation) at both soil depths, with neutral values (7.11) in the topsoil (0–30 cm) and slightly acidic conditions (5.98) in the subsoil (30–60 cm) (Table 1). At KU sites, pH values were in the alkaline range (7.78–8.04) at both soil depths. Consequently, carbonate contents were higher in the investigated soil depths at KU sites (up to 90.06 g kg⁻¹ dry soil), whereas it was very low (<0.55) at DA. Electrical conductivity was only significantly different at 0–30 cm depth and lower at DA but on a low level of non-saline soils. On the other hand, soil organic matter content was significantly higher at DA

Table 1 Soil chemical properties and extractable mineral plant macro- and micronutrients in g kg⁻¹ dry soil of samples from the investigated walnut-fruit forests, Kyrgyzstan

Property/Element	Soil depth 0–30 cm				Soil depth 30–60 cm			
	KU1—low	KU2—medium	DA—high	<i>p</i> value	KU1—low	KU2—medium	DA—high	<i>p</i> value
pH (H ₂ O)	7.78 ^b	7.93 ^b	7.11 ^a	0.007**	8.04 ^b	7.93 ^b	5.98 ^a	0.001***
SOM (%)	2.21	2.05	2.56	0.059	1.41 ^a	2.09 ^b	2.62 ^c	0.001***
EC (μS cm ⁻¹)	234 ^b	288 ^b	140 ^a	0.001***	213	221	148	0.087
Ca	5.94	13.20	7.40	0.094	3.14	4.54	3.61	0.514
K	34.81	34.50	41.28	0.208	45.74 ^{ab}	41.50 ^a	50.28 ^b	0.011*
Mg	7.91 ^a	9.68 ^b	10.10 ^b	0.001***	10.57	11.39	13.29	0.080
P	1.35	1.29	1.30	0.613	1.22	0.83	1.45	0.125
Cu	0.05	0.04	0.04	0.206	0.04	0.04	0.05	0.317
Fe	0.41	0.35	0.30	0.677	0.26	0.22	0.38	0.065
Mn	0.01	<0.00	<0.00	0.634	<0.00 ^a	<0.00 ^a	0.02 ^b	0.006**
Zn	ND	ND	ND		ND	ND	ND	

Values show means of *n*=5 soil samples per site KU1 (low altitude), KU2 (medium altitude), DA (high altitude) and depth; samples were taken from 0 to 30 cm and 30 to 60 cm soil depth under five randomly chosen trees; pH was measured in a 1:2 ratio with de-ionized water; *p* values obtained by Kruskal–Wallis test; letters indicate significant differences according to Dunn's post hoc test

SOM soil organic matter; EC electrical conductivity; ND Not detectable

in 30–60 cm soil depth, whereas it was not significantly different at 0–30 cm depth, even though the average value was higher at DA. Soil extractable mineral plant macro- and micronutrient content showed only significant differences for magnesium in the topsoil and manganese in the subsoil, with higher values at DA (Table 1). Furthermore, potassium content was slightly higher in the subsoil at DA as compared to KU2. Zinc concentrations were below the detection limit of the ICP-OES.

Physical properties of walnuts

No significant differences were found between the sites for all investigated physical walnut parameters (Table 2). Nevertheless, walnuts from DA at higher altitude were on average slightly heavier showing higher values for both, nut and

kernel mass (Fig. 1). However, values for walnut mass, *Dg* and kernel mass were not only the highest at DA with the highest mean of all three sites, but also showed the lowest values. Consequently, variability of walnut *Dg*, walnut and kernel mass was higher at DA as compared to the sites at KU at lower elevation. The proportion of shrivelled kernels was substantially lower at DA, whereas insect damage (%) was higher, again revealing differences between DA and the lower lying sites at KU. In contrast to the site comparisons, tree-specific evaluation of walnut mass, *Dg*, kernel mass proportion and kernel mass, showed significant differences ($p \leq 0.001$) between individual trees (Fig. 2, supplementary material Table S1). Accordingly, there is a higher variability between trees within a site than between sites. Furthermore, variability of the aforementioned walnut properties within one tree was also very high. Kernel colour classification

Table 2 Physical properties of nuts, chemical properties and mineral nutrient composition of walnut kernels from the investigated walnut-fruit forests, Kyrgyzstan

Physical properties	KU1—low	KU2—medium	DA—high	<i>p</i> value
Length (cm)	3.2 ± 0.1	3.1 ± 0.2	3.3 ± 0.4	
Width (cm)	2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.3	
Thickness (cm)	2.9 ± 0.1	2.9 ± 0.1	3.0 ± 0.3	
<i>Dg</i> (cm)	2.9 ± 0.1	3.0 ± 0.1	3.0 ± 0.3	
Sphericity	92.6 ± 2.8	96.1 ± 4.2	93.9 ± 5.4	
Nut mass(g)	8.3 ± 1.5	8.0 ± 1.1	9.0 ± 2.2	
Kernel mass (g)	3.6 ± 0.7	3.8 ± 0.6	4.3 ± 1.1	
KMP (%)	42.3 ± 4.3	46.4 ± 2.0	46.7 ± 1.6	
Rupture force (N)	268.2 ± 90.0	227.5 ± 54.6	273.6 ± 55.3	
Shrivelled kernels (%)	32.2 ± 0.2	20.0 ± 0.1	4.8 ± 0.1	
Insect damage (%)	2.4 ± 0.0	2.4 ± 0.0	8.0 ± 0.1	
Shell integrity (%)	24.2 ± 0.1	14.4 ± 0.1	24.8 ± 0.3	
Chemical properties				
Ash (%)	2.0 ± 0.3	1.9 ± 0.1	2.0 ± 0.3	
Moisture (%)	6.1 ± 0.0	6.6 ± 0.0	5.0 ± 0.0	
Carbohydrates (%)	14.5 ± 2.3	13.5 ± 2.0	14.1 ± 2.8	
Protein (%)	16.6 ± 2.1	15.2 ± 1.4	13.3 ± 2.7	
Fat (%)	66.9 ± 4.0	69.3 ± 3.1	70.6 ± 1.6	
TPC (mg GAE/g DW)	19.1 ± 6.6	14.4 ± 4.9	23.5 ± 7.6	
Mineral nutrient (mg 100 g ⁻¹ DW)				
Ca	117.7 ± 34.6	101.4 ± 17.7	100.0 ± 14.9	
K	422.0 ± 103.4	387.8 ± 30.1	336.6 ± 39.6	
Mg	169.3 ± 22.8	165.2 ± 12.0	149.5 ± 14.2	
P	392.8 ± 72.4	379.5 ± 29.8	339.1 ± 20.8	
S	135.7 ± 14.1 ^b	133.9 ± 9.6 ^{ab}	113.1 ± 8.7 ^a	0.02*
Cu	1.4 ± 0.3	1.3 ± 0.4	1.3 ± 0.2	
Fe	2.8 ± 0.4	2.7 ± 0.3	3.0 ± 0.6	
Mn	2.3 ± 0.8 ^b	1.9 ± 0.1 ^b	3.8 ± 0.8 ^a	0.01*
Zn	2.8 ± 0.6	2.5 ± 0.2	2.9 ± 0.4	

Values show means and standard deviations of $n=5$ trees per sampling site KU1 (low altitude), KU2 (medium altitude), DA (high altitude); *p* values obtained by Kruskal–Wallis analysis; letters indicate significant differences according to Dunn's post hoc test

Dg nut geometric mean diameter; *KMP* kernel mass proportion; values for shell integrity indicate damaged shells; *TPC* total phenolic compounds; *DW* dry weight

Fig. 1 Site-specific distribution of *Juglans regia* (L.) nut mass (a), nut geometric mean diameter (b), kernel mass (c) and kernel mass proportion (d); $n = 5$ walnut trees per site KU1 (low altitude), KU2

(medium altitude), DA (high altitude); 25 nuts per tree were examined and averaged; letters indicate significant differences according to Dunn's post-hoc test

Fig. 2 Tree-specific distribution of *Juglans regia* (L.) nut mass (a), nut geometric mean diameter (b), kernel mass (c) and kernel mass proportion (d); $n = 25$ walnut fruits per tree; 5 trees per site

KU1 (low altitude), KU2 (medium altitude), DA (high altitude) were examined; numbers (I - IV) indicate the individual trees; different letters indicate significant differences according to Dunn's post-hoc test

revealed 72–78% of the kernels exhibiting an extra light or light colour and 22–28% recorded as light amber and amber. No significant differences were found between sampling sites. Correlating kernel colour and shell integrity revealed a significant relationship with a p value of 0.047 indicating that shell damage results in darker kernel colour. Walnuts were predominantly round and long trapezoid and broad elliptic (Supplementary material Figures S1 to S3) but differed in shape between sites, trees and within one tree, again indicating the large tree specific variability of the physical walnut properties.

Chemical properties of walnut kernels

Like the physical properties of the walnuts also the investigated chemical properties of the walnut kernels did not differ significantly between the sampling sites (Table 2). However, total polyphenolic content (TPC) showed a trend of higher values in DA compared to KU2. The analysis of the mineral nutrient content of the kernels detected significant differences in sulphur content, which was on average 113 mg 100 g⁻¹ dry weight (DW) at DA at high elevation and significantly lower compared to KU1 at the lowest elevation (Table 2). In contrast, manganese (Mn) content was 3.8 mg 100 g⁻¹ DW and significantly higher at DA compared to KU. Manganese content in walnut kernels showed a significant positive relation with extractable Mn in the subsoil

($p=0.0063$). The same was true for magnesium (Mg), iron (Fe) and copper (Cu) in the subsoil (Mg: $p=-0.0063$; Fe: $p=0.0114$; Cu: $p=0.0063$) and calcium in the topsoil ($p=0.027$).

Total concentration of free amino acids in the walnut kernels was on average 195 mg 100 g⁻¹ kernel at DA and substantially higher at KU1 (314 mg 100 g⁻¹ kernel) and KU2 (414 mg 100 g⁻¹ kernel) (Table 3). Kernels of the trees sampled at DA showed remarkably lower amino acid content, except for one tree (tree DA1.1, which also had substantially lower kernel mass, see Fig. 2), as shown in Fig. 3. Arginine and glutamic acid were the dominating free amino acids. Site averages at DA, KU1 and KU2 were 77.77, 134.21 and 173.85 mg 100 g⁻¹ kernel for Arg, which, however, were not significantly different between sites. Glu, on the other hand, showed a significantly lower average for DA at 46.77 mg 100 g⁻¹ kernel as compared to KU2 with 81.34 mg 100 g⁻¹ kernel. Kernel samples from DA further showed significantly lower values for aspartic acid, histidine, valine and phenylalanine content compared to KU1 or KU2. Minor concentrations were found for tyrosine and lysine without any site-specific difference. For about half of the soil samples pH values and amino acid contents showed no or weak correlations (Table S2). However, for seven soil samples a weak to moderate correlation of pH measurements with amino acid content was found. Ten pH measurements showed a moderate or moderate to strong correlation with

Table 3 Amino acid contents of walnut kernels (mg 100 g⁻¹ DW) from the investigated walnut-fruit forests, Kyrgyzstan

Amino acid	KU1—low	KU2—medium	DA—high	p value
Aspartic acid	9.84 ± 5.46	17.92 ± 5.98	8.82 ± 8.12	0.048*
Glutamic acid	58.66 ± 7.58 ^{ab}	81.34 ± 7.45 ^a	46.77 ± 8.62 ^b	0.004**
Serine	9.74 ± 4.58	13.55 ± 7.05	6.23 ± 3.07	0.10
Histidine	21.06 ± 7.64 ^{ab}	23.20 ± 8.94 ^a	9.11 ± 4.46 ^b	0.032*
Glycine	3.37 ± 0.93	12.95 ± 18.59	2.58 ± 1.17	0.11
Threonine	17.48 ± 9.16	17.03 ± 5.56	12.58 ± 2.42	0.34
Arginine	134.21 ± 59.73	173.85 ± 35.63	77.77 ± 133.31	0.14
Alanine	8.60 ± 3.59	14.67 ± 12.56	4.89 ± 5.12	0.13
Proline	5.77 ± 3.97	5.59 ± 3.78	6.11 ± 2.85	0.85
Tyrosine	3.37 ± 2.44	2.33 ± 1.89	1.47 ± 1.07	0.48
Valine	11.53 ± 5.53	15.92 ± 8.37	4.68 ± 5.13	0.048*
Methionine	6.32 ± 5.76	5.08 ± 5.75	4.13 ± 5.85	0.47
Cysteine	4.04 ± 4.46	5.48 ± 4.84	4.66 ± 5.15	0.70
Isoleucine	5.83 ± 3.24	8.09 ± 6.45	3.94 ± 4.41	0.20
Leucine	5.29 ± 4.86	8.29 ± 7.33	4.04 ± 3.93	0.54
Phenylalanine	9.14 ± 4.65 ^a	6.38 ± 2.26 ^{ab}	3.38 ± 2.82 ^b	0.039*
Lysine	2.19 ± 1.50	3.59 ± 2.38	2.59 ± 2.60	0.29
Total	314.01 ± 102.67	414.25 ± 53.76	195.10 ± 172.63	0.12

Values show means and standard deviations of $n=5$ trees per sampling site KU1 (low altitude), KU2 (medium altitude), DA (high altitude); p -values obtained by Kruskal–Wallis analysis; letters indicate significant differences according to Dunn's post-hoc test

DW dry weight

Fig. 3 Total free amino acid content of *Juglans regia* (L.) kernels; n = 5 trees per site KU1 (low altitude), KU2 (medium altitude), DA (high altitude); significant differences were found by applying Kruskal-Wallis test; Dunn's post-hoc test could not confirm significances

amino acid content with an overall weak to moderate correlation of total amino acid concentration.

Discussion

We hypothesized that the physical and chemical walnut and kernel properties measured in our study, in particular total phenolic compounds (TPC), vary between the three sites as caused by differences in altitude and associated environmental conditions, especially average air temperature. However, no significant differences were detected between the sites for almost all walnut properties, except for some mineral nutrients. In contrast, significant differences between individual trees independent of the site were found, which indicates that intraspecific variability has a greater impact on walnut properties than abiotic factors. These results are supported by some sources in the literature. Martínez et al. [12] summarized information about genetic and environmental effects on walnuts showing that physical parameters such as walnut shape, size, weight, kernel colour and shell thickness are attributed to allelic variation. Similar observations were made by Roor et al. [5] showing additionally that sphericity is rather subjected to genetic drift than to environmental factors. Rabadán et al. [31] in their study showed, that cultivar defines oil content and some fatty acids, which could not be measured in our study. However, the authors also showed, that annual weather conditions strongly affected other nutritional properties of walnuts, such as protein and mineral content. As walnuts of the present study were all treated in the same way concerning drying, transport and analysis and do not differ among sampling sites, significant differences between trees independent of the site origin can be attributed to genetic variability. However, Roor et al. [5] found rather low allelic richness in Kyrgyz walnuts compared

to Middle East or South Asian walnuts, so that one would assume only slight differences in chemical composition amongst Kyrgyz nuts. Future studies need to elucidate the relationship between genetic variability and walnut quality in Kyrgyzstan.

Walnut oil and phenol content is not only coded by genotype but may also be influenced by environmental conditions and therefore can be affected by irrigation, storage and processing [12]. These important properties thus need to be evaluated for the Kyrgyz walnuts in future studies. Regarding fat content, Kyrgyz walnut kernels were within the upper range of contents detected in literature; Savage [32] found 62–70% fat in walnuts from New Zealand, Europe and the USA. Turkish cultivar fat content ranged from 49 to 65% [33]. Ash and carbohydrate content were comparable to literature, where values ranged from 1.2 to 2.8% and 13.8–17.2%, respectively. Cultivars from New Zealand, Europe, the USA and Turkey featured 13–20% protein content [32, 33], which is in the range of the values found in our study. In most studies, a conversion factor of 6.25 was used to calculate protein content; thus, Kyrgyz samples were slightly higher in protein, once taking the different conversion factor into account.

The Kyrgyz walnuts in our investigation showed quite high moisture contents, exceeding the international quality standard of a maximum of 5.0%, which was potentially caused by a short drying period. Fuentealba et al. [15] suggested that higher moisture contents accelerate oxidation reactions leading to browning of the kernel. Furthermore, it is assumed that brown kernel colour indicates low antioxidant capacity as reflected by low content of TPC [15]. Even though in our study no significant differences for TPC were found between sites, a similar trend as indicated in the literature was observed. The KU2 composite kernel samples contained the lowest TPC content, while having higher

residual moisture (6.6%) and a higher proportion of light amber and amber walnuts (28%) when excluding shrunken kernels. DA showed the highest TPC values at the lowest residual moisture (5%). Furthermore, no amber kernels were found, and proportion of light amber kernels amounted to only 21.8% leaving a large proportion light kernels from DA. Moreover, the proportion of darker kernels was related to walnut shell integrity, yielding higher oxygen availability in the kernel environment and therefore supporting oxidation. Nonetheless, the Kyrgyz walnuts were overall higher in TPC compared to values recorded for Chilean walnut kernels ranging only from 5.7 to 12.7 mg GAE g⁻¹ DW [15] as compared to average values above 14 mg GAE g⁻¹ DW in our study but in the range of values reported elsewhere [34].

When comparing walnut and kernel properties of Kyrgyz walnuts with values published in literature, it can be seen, that for kernel mass they fall within the same weight range as genotypes from Turkey, weighing 6.6–8.9 g per nut [35]. However, according to Mamadjanov [6] cultivated ecotypes from Ukraine and Russia were clearly higher in mass ranging from 11.8 to 16.2 g, respectively. Kernel masses of this study rather fall into the lower range of values found by Patraş and Dorobanţu [36] who determined 3.0–8.0 g per kernel. Concerning kernel mass proportions cultivars from Russia, Ukraine, the USA, China and Turkey obtained higher values ranging from 46 to 64% [35]. Kyrgyz walnut kernel proportions were higher or equal to wild Moldavian and Romanian cultivars [36, 37]. Even though genotype has a strong effect on walnut properties, Roor et al. [5] found walnut density correlating with altitude, latitude and longitude and Diaz et al. [18] reported phenotypic variation of Spanish walnut cultivars due to latitude designating light and climatic conditions. Consequently, future studies need to consider the interactive effects of genotype and environmental factors, such as seasonality, temperature range or water availability [10]. Ramos et al. [38] found that water stress affected walnut quality leaving a higher proportion of kernels shrunken. In our study, lower temporal water availability may have caused the high proportion of shrivelled kernels at the lower lying sites KU1 (32%) and KU2 (20%) as compared to DA (5%). This is supported by Mamadjanov [6] who found Kyrgyz walnut quality being negatively affected by drought. We cannot exclude topographic and microclimatic conditions differing between the investigation sites, as this was not explicitly measured in the present study. At the highest altitude (DA) kernels were primarily damaged by insects (8% of the whole samples) and less by kernel shrivelling. Further evidence for a site effect is that, even though not significant, DA kernels show considerably lower total free amino acid contents, except of one tree (DA1.1). Hildebrandt et al. [39] explain that during stress periods such as drought, protein degradation in plants takes place leading to an increased total free amino acid pool. Fuentealba et al. [15] suggested that accumulation of soluble

amino acids indicates poor conversion to other compounds. Walnut kernels from KU1, KU2 and the individual tree DA1.1 show higher proportions of dried kernels likely caused by pronounced drought stress. The total free amino acid pool of the kernels thus could be a result of trees suffering from low water availability during fruit development. However, logistic regression does not show a correlation between amino acid content and proportion of shrivelled kernels in the samples.

Mineral nutrient content of Kyrgyz walnut kernels investigated in this study was within the range of values reported in the literature [9, 13]. Only manganese (Mn) was about one third and one half lower for kernels from KU1 and KU2, respectively, when comparing to values published in the national nutrient database for standard references [3, 40].

In contrast to walnut kernel parameters, sampling sites differed significantly in some of the soil characteristics, such as pH, EC and SOM. Lower pH values were measured especially in the subsoil at DA, which goes along with a lower carbonate content. This corresponds well to higher extractable manganese (Mn) content, which further relates to a significantly higher Mn content in the walnut kernel at DA as compared to the KU sites. Mn content of walnut kernels was in the range of values reported earlier [8]. These results show that soil conditions influence the availability of nutrients for walnuts, which actually is reflected in the nutrient concentration of the walnut and the relationship between some of the mineral content measured in the walnut kernel samples and the Machigin soil extract. In an earlier study, total elemental concentrations in the soil were not related to content of the same element in walnut kernels [8]. Our results indicate, that extractable soil nutrients as affected by site conditions (e.g. pH) influence nutrient composition of walnut kernels supporting our hypothesis. This, however, is only true for some of the mineral elements or micronutrients.

Soil organic matter can store water and nutrients. The values in the topsoil at the three sites were not different, however at DA the subsoil showed a significantly higher SOM content. This may have contributed to larger water storage and availability to the walnut plants and explain the lower proportion of shrivelled kernels at DA compared to KU. SOM values were in general at the lower end of values reported by Sakbaeva et al. [41], who found values between 2.4 and 12.5% in the topsoil and 1.2–6% in the subsoil of Kyrgyz walnut forests.

Correlations were found between soil pH and free amino acids in the kernels. The question if soil pH considerably influences the free amino acid pool of walnut kernels is not easy to answer as research not yet investigated on how the soil pH influences free amino acids in plants in general or particularly in its fruits and seeds. Hildebrandt et al. [39] explains that the free amino acid pool is a result of protein catabolism which rather depends on a specific stage in the

lifecycle of a plant or on severe environmental stress than on moderate differences in soil pH. Even though a correlation was found between the two examined parameters, it is unlikely that soil pH impacted the free amino acid pool found at the time of the investigation. Soil pH may influence the bound pool of amino acids during fruit ripening but as literature reveals no explanation, future research may elucidate this.

Conclusions

Our study shows that variability of physical and chemical walnut and kernel properties is higher between individual trees than between sites. This indicates, against our hypothesis, that the potential site-specific environmental effects, such as a difference in temperature due to altitude, are superimposed by genetic variability in the investigated Kyrgyz walnut forests. Some soil extractable elements can reflect mineral composition of walnut kernels and some free amino acids are affected by site conditions for unknown reasons. Consequently, further verification is required. Future studies also need to evaluate oil and fatty acid content of walnuts in relation to genetic variability in the Kyrgyz walnuts to allow the evaluation for oil extraction. We conclude that changes in average temperature as caused by future climate change may not be reflected in average changes of the assessed quality parameters in the Kyrgyz walnuts. However, shrivelled kernels found in large proportions at two of the investigated sites reveal environmental stress, potentially drought, to some of the walnut trees, which may become more frequent in the future and need to be considered in tree selection for forest regeneration.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirement This paper does not contain any studies with human or animal subjects.

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