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Influence of Various Nitrogen Applications on Protein and Amino Acid Profiles of Amaranth and Quinoa

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The effect of nitrogen application levels (0.16 and 0.24 g N kg^{-1} soil) on seed proteins and their amino acid compositions of amaranth (*Amaranthus* spp.) and quinoa (*Chenopodium quinoa* Willd) was studied. Total proteins of amaranth and quinoa had high contents of lysine (6.3−8.2 g 100 g^{-1} protein) but low contents of methionine (1.2−1.8 g 100 g^{-1} protein). Seed proteins were fractionated on the basis of different solubility in water, saline, and buffer as albumin-1 (Albu-1), albumin-2 (Albu-2), globulin (Glob), and glutelin (Glu) and were identified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Albu-1 was high in lysine (5.4−8.6 g 100 g^{-1} protein), while Albu-2, which is a part of storage proteins, had a high leucine content (7.2−8.9 g 100 g^{-1} protein) as an effect of different nitrogen application levels. Glu fractions were well-balanced in their essential amino acids with the exception of methionine. In conclusion, nitrogen application can be used for the nutritional improvement in human diet by increasing and maintaining protein and essential amino acid contents.

KEYWORDS: Amaranth; quinoa; protein; amino acid; nitrogen levels

INTRODUCTION

There are some questions regarding the prevalence of allergies to plant food. Prevalence estimates were categorized by food item and method used (food challenges, skin prick test, serum IgE, and parent/self-reported symptoms), complemented by appropriate meta analyses (*1*). The prevalence of sensitization against any specific plant food item assessed by a skin prick test is usually <1%, whereas sensitization assessed by IgE against wheat ranges as high as 3.6% and against soy as high as 2.9%. Persons suffering from celiac disease (CD) must avoid foods containing gluten or those contaminated with wheat, barley, or rye. Rice-, corn-, or quinoa-based foods as gluten-free plants are the safest for celiac patients (*2*). Fortification of gluten-free products with folic acid or enrichment of these products with nutrient-dense fractions of cereals naturally free from gluten (such as buckwheat, quinoa, amaranth, or millet) can be of interest, because the major storage proteins in amaranth and quinoa seeds are globulins (*3, 4*). According to recent reports (*1, 2*), such allergens have not been observed in amaranth (*Amaranthus* spp.) and quinoa (*Chenopodium quinoa* Willd). Therefore, they may be used as an alternative source for nonallergenic food products. Amaranth and quinoa are protein-rich, and globulins are the major storage proteins in amaranth and quinoa seeds and have a better balance in the amino acid composition than other cereals such as wheat, maize, and oat. Therefore, the sum of essential amino acids (EAA) in amaranth and quinoa seeds is closely related to the recommended amino acid pattern of the FAO/WHO standard (*5*). Nitrogen supply has a dominant effect on protein accumulation in the seed.

Hayati et al. (*6*) reported that in soybean seeds, the nitrogen accumulation and concentration were related to the nitrogen concentration in the media under in vitro culture conditions. Paek et al. (*7*) showed that raising the soybean seed protein concentration through enhanced plant nitrogen affected the quality of seed storage proteins. Nitrogen was translocated mainly via phloem in the form of amino acids. Higher protein contents in rapeseeds were correlated with higher amino-nitrogen translocation rates in the phloem (*8*). Protein fractions in wheat grains (*9*) and amino acid composition changes in rye and oat grains (*10*) related to nitrogen application levels were reported. As can be concluded from the cited literature (*7*−*10*), the information on the use of different nitrogen levels was extensively done using different plant species. Nitrogen played an essential role in the nutrient relationship between plants and pathogens. Some studies (*11*) reported that the nitrogen-mobilizing plant metabolism that occurs during abiotic and biotic stress could be a "slash-and-burn" defense strategy. A direct

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effect of nitrogen supplementation on amino acids and protein distribution was discussed (*11*). The research on transgenic barley plants with altered storage protein distribution exhibited significant changes in amino acids (*11*). Amino acids metabolism in quinoa (*12*) was investigated by the control of lysine, threonine, and methionine, as well as by other nitrogen-containing compounds (nitrate, nitrate reductase activity). However, information about the effects of nitrogen fertilizer on the protein properties of amaranth and quinoa was very limited. The purpose of this study was to separate and then to determine the contents of albumins, globulin, and glutelin fractions in amaranth and quinoa seeds as well as their amino acid compositions depending on nitrogen supply. The results of this research are new for these plants species.

MATERIALS AND METHODS

Plant Materials. Two amaranth [Bärnkraft (*A. cruentus*) and K432 (*A. hypochondriacus* × *A. hybridus*)] and quinoa (Faro and Tango) varieties were cultivated and applied with three treatments of NH_4NO_3 fertilizer (0.16 and 0.24 g N kg^{-1} soil). To reach 0.24 g N kg^{-1} soil, top dressing nitrogen fertilizer as 0.08 g N kg^{-1} soil was added to one of the 0.16 g N kg^{-1} soil treatment at the flowering stage. Phosphorus and potassium supplies were used as 0.12 and 0.16 g kg^{-1} soil, respectively (adjusted with $CaHPO_4$ and K_2SO_4). Whole mature seeds of amaranth and quinoa were ground on a laboratory mill 120 (Perten Instruments AB, Huddinge, Sweden) through a 60-mesh screen and stored at 4 °C in airtight plastic bottles until use.

Protein Extraction and Determination. Protein fractions were extracted stepwise using water, saline, and different buffers, and then, the protein contents in each fraction were determined according to the following methods (*4, 13*−*17*).

Protein Characterization with Sodium Dodecyl Sulfate Polyacrylamide−**Gel Electrophoresis (SDS-PAGE).** SDS-PAGE was performed according to Laemmli (*18*) using a gel concentration of 10% with some modifications as previously described (*13*). The protein molecular weight (MW) was identified by SDS-PAGE using the Biometra Mini-Power Pack 040-100 and PP 2000 with glass plates (6.6 cm × 7.7 cm) (Biometra, Germany). Protein standards mixture IV (Merck, Germany) was applied to determine protein subunit molecular masses in kDa: cytochrome C (12), myoglobin (16), carboanhydrase (30), ovalbumin (42), albumin (66), and ovotransferrin (78). The MWs of unknown proteins were estimated by SDS-PAGE by plotting the log of the MW of standard proteins vs their electrophoretic mobility.

Amino Acid Composition. The amino acid composition was done by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (*14, 19*). Samples of extracted proteins were hydrolyzed with 6 M HCl and 3% phenol solution under vacuum and heated at temperatures of 60 °C for 24 h. The samples were then dissolved in 100 μL of HCl (20 mM) and filtered through a 0.45 μm filter.

The sample was injected into a Multi-Pump Gradient HPLC system (Waters, Milford, MA) with a vertex precolumn spherimage ODS2; 5 μm, 5 mm × 4 mm and a vertex separation column, spherimage-80 ODS2; 5 μm, 4.6 mm × 150 mm (Knauer, Germany). The temperature was maintained in the column of 40 °C at the flow rate 1 mL/min. The Millenium chromatography manager system (Waters, Milford, MA) was applied to evaluate the amino acids content. A scanning fluorescence detector was used at an excitation of 250 nm and emission of 395 nm. The gradient program was provided with sodium acetate phosphate buffer and acetonitrile/water solution (60/40; v/v). Tryptophan was not determined. The results are given as g amino acid (AA) 100 g^{-1} protein. An internal standard was used for quantifying amino acids by high-performance liquid chromatography (HPLC).

Statistical Methods. Split-plot in completely randomized design (CRD) with three replications was used. Data were evaluated by analysis of variance, and means were tested by least significant difference (LSD). The p values of <0.05 were considered as significant. The statistical analyses were performed by using Statistix 7.0 (Analytical Software, Tallahassee, FL). Main plots were cultivars, and subplots were the nitrogen levels.

Table 1. Effect of Nitrogen Fertilizer on Seed Protein Fractions[a]

		fraction (%)[b]				
plant species	varieties	Albu-1	Albu-2	Glo	Glut	Rest[c]
amaranth	Brnkraft	10.5 b	3.6 b	51.6 a	5.6 b	28.7 b
	K432	6.2 a	2.1 a	51.0 a	8.2 c	32.5 b
quinoa	Faro	7.0 a	6.2 c	60.2 b	3.2 a	23.4 a
	Tango	12.0 c	1.4 a	51.4 a	5.9 b	29.3 b
nitrogen levels[d] (g N kg^{-1} soil)						
0		10.7 b	3.7 a	51.0 a	6.2 a	28.5 a
0.16		8.5 a	3.7 a	54.6 a	6.1 a	27.2 a
0.24		7.4 a	2.7 a	55.1 a	5.0 a	29.7 a

[a] The mean values in columns with different superscript letters are significantly different ($P > 0.05$). [b] Protein fractions: Alb-1, albumin-1; Alb-2, albumin-2; Glo, globulin; and Glut, glutelin. Expressed as total protein percentage. [c] The protein content of the rest was determined as nitrogen with the Dumas combustion method. [d] Average values of all plant species and cultivars.

RESULTS AND DISCUSSION

Protein Fractions. The distribution of the single fraction within the proteins was mainly dependent on the cultivars (**Table 1**). The average ratio of Albu-1:Albu-2:Glob:Glut:rest was 8.9:3.3:53.6:5.7:28.5. Glob was the main protein fraction followed by Albu-1. In this study, the Alb and Glob were higher than those previously reported by Bressani and Garcia-Vela (*3*). The different values may be caused by the variation of the plant material, methods, and amount of the sample used for extraction.

Albu-1 significantly decreased after nitrogen application. Albu-1 content was negatively correlated with the Glob and was affected during the seeding development stage. According to Tabe et al. (*20*), the down-regulation of prolamins in *opaque 2* mutants in maize was associated with a compensatory increase in nitrogen storage in other seed protein fractions.

The prolite functional proteins (gluten) are mostly accumulated in grain mainly during the cell division stage, whereas the storage proteins (gliadins and glutenins) are accumulated mainly during the filling period (*9*). Martre et al. (*21*) found that the accumulation of storage proteins is significantly enhanced by nitrogen supply, whereas functional protein content is less affected.

Electrophoresis Patterns. A variation in all protein fractions of the amaranth cultivars, but not of quinoa, was found (**Figure 1**). This result in amaranth shows variations in some minor bands and is in agreement with the previous findings (*4, 13, 14*). Nitrogen application did not change the electrophoresis patterns of the studied cultivars.

Albu-1 differences in protein subunit patterns of amaranth varieties 'Bärnkraft' and 'K432' have occurred (**Figure 1A**). Albu-1 of 'Bärnkraft' had a lower MW than 'K432'. 'Bärnkraft' contained three major subunits at 28, 30, and 33 kDa, respectively, while 'K432' consisted of four major subunits with MW of 26, 28, 30, and 34 kDa. These results were similar to the protein patterns of amaranth reported by Drzewiecki et al. (*22*).

For quinoa, Albu-1 (**Figure 1A**) showed a wide range of major protein subunits within the range of 25−83 kDa. Both varieties had similar major protein bands at 25, 28, 36, 39, 78, and 83 kDa. Differences in the major subunits between 'Faro' and 'Tango' were at 47 and 57 kDa. Protein patterns of quinoa were similar to soybean, which had major subunits under 30 kDa as well as above 42 and 78 kDa (*13*).

The major protein subunits in Albu-2 of amaranth (**Figure 1B**) are divided into three groups with the range of 22−25,

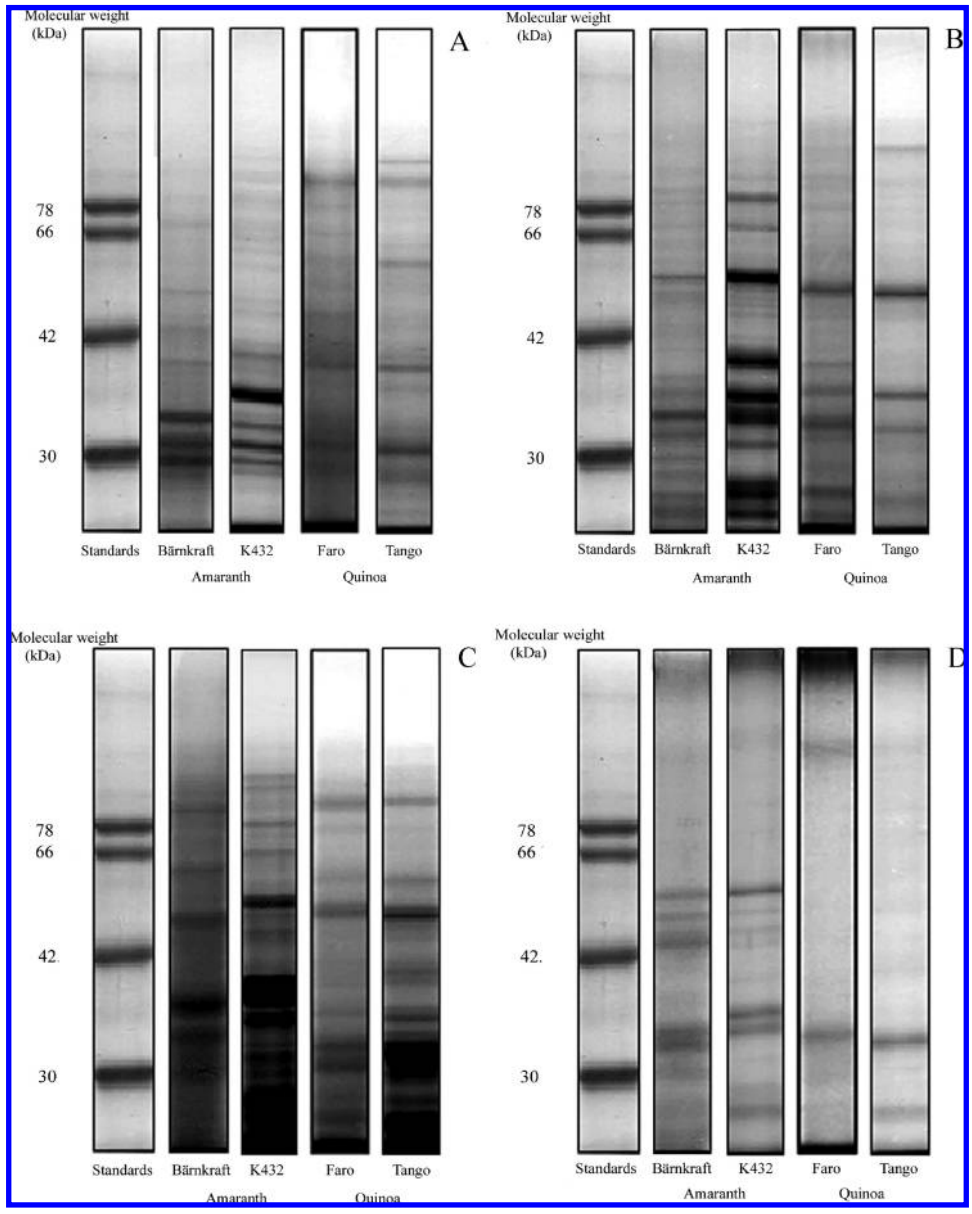

Figure 1. SDS-PAGE patterns of Albu-1 (**A**), Albu-2 (**B**), Glob (**C**), and Glut (**D**) of amaranth and quinoa at all levels of nitrogen fertilizer.

31−39, and 51−54 kDa. The major protein subunits in amaranth variety 'Bärnkraft' were 31 and 57 kDa. The 'K432' variety contained several major protein subunits when compared with 'Bärnkraft' variety (22, 28, 31, 34, 39, 53, 64, and 72 kDa, respectively). Both varieties of quinoa consisted of four major subunits with MWs of 23, 31, 35, and 52 kDa. The predominant protein subunits in Glob of 'Bärnkraft' (**Figure 1C**) were 29, 34, 38, and 52 kDa. The 'K432' variety had several low MW protein subunits in the range of 22−26 kDa and four major protein subunits with MWs of 30, 34, 38, and 64 kDa. In this study, the protein patterns in amaranth seeds consisted of the higher MW proteins than that reported by Gorinstein et al. (*4*). Glob electrophoretic profiles of both quinoa varieties were similar in the major protein subunits. There were distinct protein subunits within the range of 29−32 kDa and four other protein bands at 23, 35, 58, and 78 kDa. As was mentioned in the Introduction that amaranth and quinoa are dicotyledonous plant whose major seed storage proteins are globulins and glutelins. A unique feature of amaranth and quinoa seeds is the presence of Albu-2, which is extractable with water only after an exhaustive extraction of Glo and Albu-1.

The exact relationship between the Albu-2 fractions and the 11S proteins was studied as a hypothesis that the Albu-2 fraction could consist of a nonprocessed 11S globulin (proglobulin) (*15*). In such a way, Albu-2 can be related to a storage protein as the globulin fraction. A 55 kDa component of Albu-2, which specifically cleaved into 38, and 17−15 kDa polypeptides, was observed. The presence of unprocessed 11S precursors in mature amaranth seeds of 57 kDa (*15*) corresponds with our, results of the MWs. Similar data were shown in relation to quinoa, as 11S globulins and 2S albumins are responsible for the relatively high protein content and ideal amino acid balance of the quinoa seed (*16*). Additionally, the phylogenetic relationships between quinoa and 49 other species for the well-conserved 11S basic subunit were reported (*16*).

Similar patterns of protein bands in amaranth 'Bärnkraft' and 'K432' were shown also for Glut. The major protein subunits of 'Bärnkraft' were found at 32, 34 48, 53, and 57 kDa. For 'K432', the major protein bands at 24, 32, 34, and 55 kDa were detected. The patterns of amaranth are in accordance with Gorinstein et al. (*4*). Quinoa had less protein bands as compared

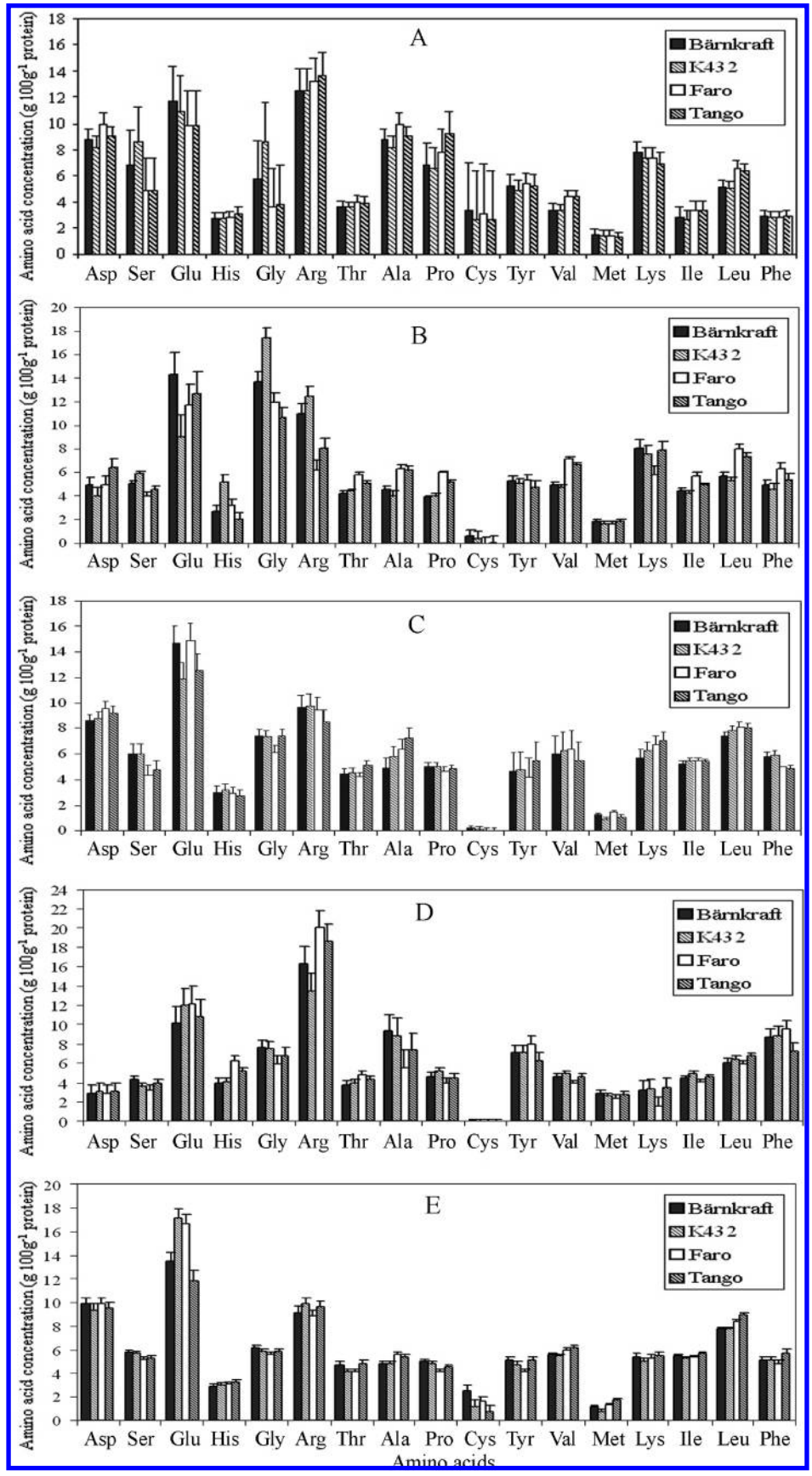

Figure 2. Amino acid concentrations in seed (**A**), Albu-1 (**B**), Albu-2 (**C**), Glob (**D**), and Glut (**E**) of amaranth and quinoa. Bars indicate a LSD of 0.05.

to amaranth. The variety 'Faro' consisted of two major protein subunits with MWs of 32 and 94 kDa, while 'Tango' contained the two subunits of 25 and 32 kDa (**Figure 1D**).

Amino Acid Composition. In **Figure 2**, the amino acid composition of amaranth and quinoa seeds is presented. Analyzed were the following amino acids: aspartic acid (Asp),

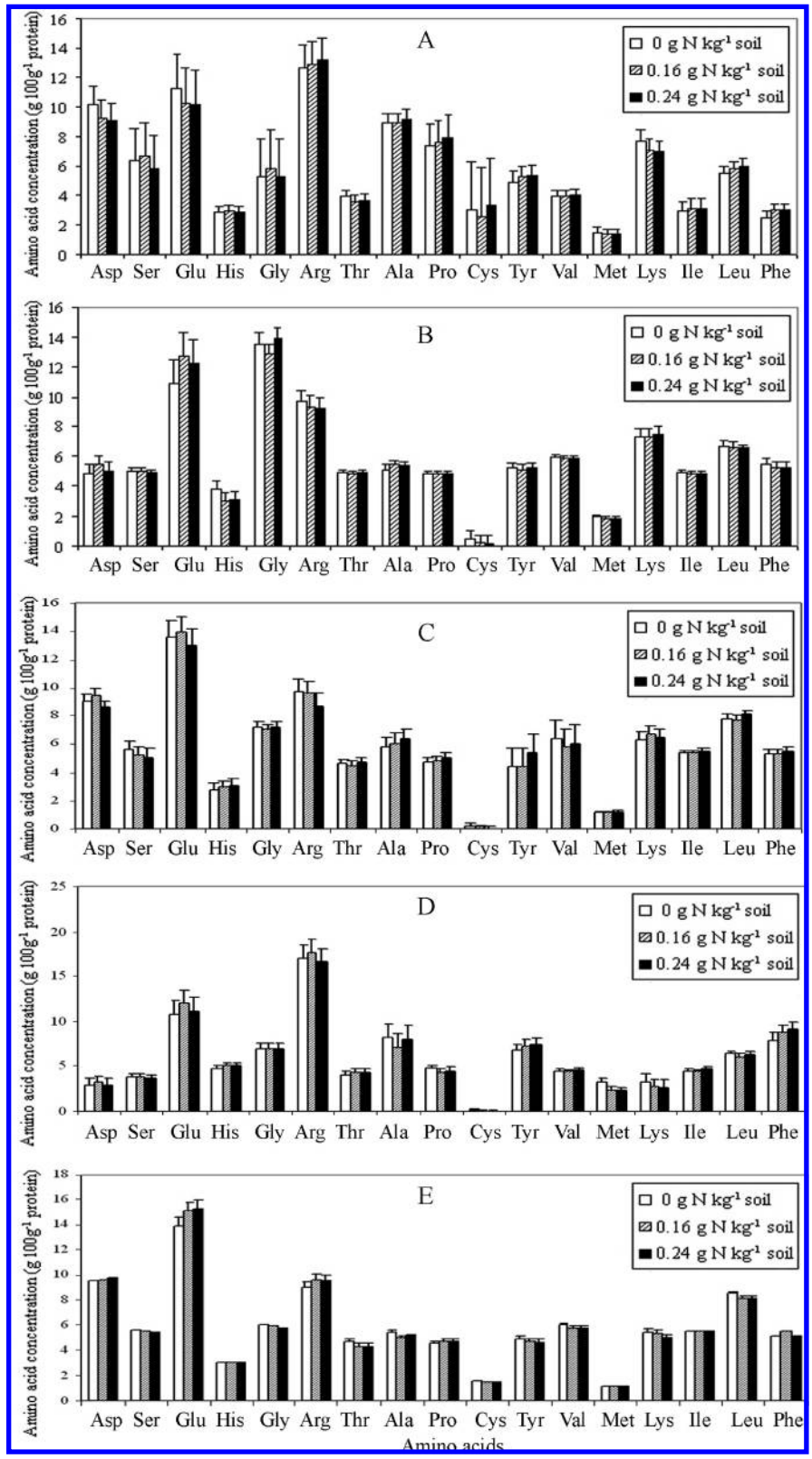

Figure 3. Amino acid concentrations in seed (**A**), Albu-1 (**B**), Albu-2 (**C**), Glob (**D**), and Glut (**E**) of amaranth and quinoa at different rates of nitrogen applications (average of all species and cultivars). Bars indicate a LSD of 0.05.

serine (Ser), glutamic acid (Glu), histidine (His), glycine (Gly), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu), and phenylalanine (Phe). Amaranth flour contained significantly lower amounts of Val and Leu than quinoa. The sums of essential amino acids (EAA) in quinoa were higher than amaranth (45.8 and 41.5 g 100 g^{-1} protein, respectively).

The amino acid concentrations response to nitrogen application is shown in **Figure 3**. Pro and Glu contents were negatively correlated with Arg content. Plants take up nitrogen preferably as ammonia and then convert it into glutamine (Gln) and Glu.

These two amino acids act as nitrogen donors for the Pro and Arg biosynthesis (*23*). Similar conclusions were reported that the amino acid profile analysis in leaves and roots of quinoa also indicated an important role of soluble glutamine as a nitrogen-transporting compound (*12*). In this report, only protein glutamine was determined. Glutamine accumulation correlated with the accumulation of cytosolic glutamine synthetase (*11*). Other researchers suggest that several amino acids, and in particular L-Gln and L-Asn, promote the growth of *Arabidopsis*, and increased expression of specific amino acid transporters enhances growth on amino acids. The efficiency by which transgenic plants exploit D-amino acids illustrates how plants can be engineered to utilize specific N sources otherwise inaccessible to them (*17*).

The Phe content increased from 2.5 to 3.0 and 3.0 g 100 g^{-1} protein when applied at 0, 0.16, and 0.24 g N kg^{-1} soil, respectively. Bullman et al. (*24*) observed in barley that the Phe content was increased proportionally to the rising rates of nitrogen fertilizer supply. Amaranth and quinoa contained higher amounts of Lys (6.3−8.2 g 100 g^{-1} protein) than wheat, maize, rice, and barley (1.98, 3.52, 4.0, and 3.08 g 100 g^{-1} protein) (*25*). The high concentration of lysine observed in quinoa seeds is possibly due to a combined effect of increased lysine, synthesis, and accumulation in the soluble form and/or as protein lysine (*12*).

This value is also higher than recommended (5.5 g 100 g^{-1} protein) in the FAO/WHO standard (*5*). The content of Met, lower than FAO/WHO standard, might be affected by Cys synthesis (*26*). However, the total EAA content was not changed after nitrogen application (**Figure 3A**). For the human diet, nitrogen fertilizer application might be an advantage to improve the nutritional values by increasing protein content and maintaining most of essential amino acids content.

In the Albu-1 of amaranth and quinoa, the amino acid compositions were different (**Figure 2B**). However, EAA contents of amaranth and quinoa were not significantly different when compared to each other (average 49.1 g 100 g^{-1} protein). Albu-1 contained more Gly than other fractions. On the other hand, the concentration of Gly found in this study was considerably higher than that (4.7 g 100 g^{-1} protein) reported by Bressani and Garcia-Vela (*3*). After nitrogen application, the EAA content decreased from 50.3 to 48.7 and 48.6 g 100 g^{-1} protein, respectively, and significantly decreased when the rates of nitrogen fertilizer increased. The Met content was lower than FAO/WHO standard (1.8 and 3.5 g 100 g^{-1} protein, respectively). Phe increased along with the increasing rates of nitrogen application (**Figure 3B**).

Albu-2 (**Figure 2C**) differed in amino acids composition depending on cultivars and nitrogen application. The average value of EAA was 49.1 g 100 g^{-1} protein. Amaranth had a higher content of Ser than quinoa. It was found that the concentrations of Asp, Arg, and Leu were affected by nitrogen application (**Figure 3C**). However, only the Arg content was distinctly decreased after increased nitrogen fertilizer rates. The Met content was low in this fraction. Albu-2 fractions had higher contents of Leu than that of Albu-1 and Glob (**Figure 3C**).

The amino acid composition in Glob (**Figures 2D** and **3D**) was influenced by cultivars and nitrogen fertilizer. Quinoa had significantly higher EAA contents than amaranth (57.8 and 53.3 g 100 g^{-1} protein, respectively). The content of sulfur containing amino acid Met was decreased along with the rates of nitrogen application, while Ile and Phe were increased (**Figure 3**). Nakasathien et al. (*27*) reported that increased nitrogen supply affected the sulfur amino acid synthesis. The Phe content was high in this fraction. Lys and Cys contents were lower than in the other fractions. Amaranth and quinoa contained less Lys than *Phaseolus vulgaris* (2.88 and 10.75 g 100 g^{-1} protein, respectively) when compared with data previously reported by Chagas and Santoro (*28*). Shewry et al. (*29*) proposed that seed Glob fractions were deficient in Cys and Met. In contrast, Gorinstein et al. (*13*) showed in their study that Glob was rich in Met and Cys, Ile, and Val. The Phe content was increased in response to increasing rates of nitrogen supplied. Therefore, increasing the Phe content of amaranth and quinoa flour should be affected by Glob fractions.

Glut of amaranth and quinoa (**Figures 2E** and **3E**) varied also in amino acid composition. Quinoa 'Tango' had the highest EAA content when compared with the other cultivars (51.5 and average 47.0 g 100 g^{-1} protein, respectively). Glut had a high content of Leu. It was found that the Lys content was higher than soybean in a previous report by Gorinstein et al. (*14*). However, the Lys content in Glut was lower than Albu-1 and Albu-2. Alb was the major source of lysine, which is synthesized during seed development (*29*).

In conclusion, nitrogen application affected protein fractions and amino acids composition of amaranth and quinoa, but the amount of essential amino acids was not changed by nitrogen application. During seed development, the decrease of Albu-1 was shown. Albu-1 had a high Lys content, while Albu-2 was high in Leu. Glob contained higher amounts of essential amino acids than the other fractions but a lower content of Lys. Glut was well-balanced in essential amino acids with the exception of Met. Nitrogen fertilizer application could be an advantage for improving the nutritional values of the human diet by increasing protein contents and maintaining essential amino acid contents.

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