Effect of nitrogen fertilization on metabolisms of essential and non-essential amino acids in field-grown grain maize (*Zea mays* L.)

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ABSTRACT

In two-year field experiments, nitrogen (N) in the form of urea (0, 120 and 240 kg N/ha) was applied to grain maize (*Zea mays* L.) hybrid KWS 2376. The two-year mean content of total grain N at harvest was 1.54%. The highest N dose reduced most of the 17 amino acids (AA) analysed in the grain compared with the other treatments. Possible reasons for this could be an adverse effect on the tricarboxylic acid cycle or deficiency of carbon skeletons for the assimilation of NH_4^+ into amides and amino acids. The content of the limiting amino acid lysine was not influenced by N fertilisation, with a mean two-year content of 2.02 mg/g DM. Taking into account the differences in fertilisation, the effect of the year was seen in the maximal accumulation of amino acids serine, proline, methionine, threonine, arginine and lysine. Increasing rates of nitrogen reduced the accumulation of asparagine and glycine, and, on the contrary, increased the accumulation of tyrosine. Nitrogen rates have a significant effect on the maximal accumulation of valine, isoleucine, leucine, phenylalanine, histidine, cysteine and alanine and appeared as early as after the first increased rate of nitrogen (120 kg N/ha).

Keywords: corn; effect conditions year and nutrition; plant uptake; regulation biosynthesis amino acids; aleurone layer in seeds; metallothionen-like proteins; senescence

Maize (*Zea mays* L.) plays a very important role in human and animal nutrition (Lošák et al. 2010). The major factors controlling crop quality are genetically determined. Lysine is the limiting amino acid for all cereals and the amount varies between species, being highest in oats and rice and lowest in wheat and maize (Shewry 2007). Exogenous factors can considerably influence the concentrations of organic compounds in plant organs of relevance to produce quality.

Nitrogen (N) nutrition is one of the major exogenous factors limiting growth and production of crop plants. The content of amino acids, important N-containing compounds, in plant biomass is also affected by N nutrition (Neuberg et al. 2010a, Pavlík et al. 2010a). Nitrate and ammonium are the major sources of N for plants. Ammonium-supplemented plants often show a higher concentration of amino acids than nitrate-supplemented plants, while higher N supply also causes an overall increase in

amino acid content (Atanasova 2008). According to Tilsner et al. (2005), under low N supply, the amino acid contents are comparable at all leaf ages and decrease slightly from young to mature leaves.

Most of the NH_4^+ taken up by roots is assimilated in the roots and translocated in the form of amino acids and amides to the shoots. Assimilation of NH_4^+ into the roots requires carbohydrates to be translocated from shoots to roots to provide the C skeletons and energy (ATP and NADPH) for the NH_4^+ assimilation process (Mengel and Kirkby 2001).

The assimilation of nitrate and ammonium gives rise to ammonia (NH_3). For ammonia assimilation, free enzymes are important: glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT). The efficiency of GS may differ between plant species. Glutamic acid formation is a port of entry for N into organic compounds and occurs in the chloroplasts or mitochondria (Mengel and Kirkby 2001).

The aim of this study was to examine the effects of varying N fertilisation in the form of urea on the composition of amino acids synthesised in maize.

MATERIALS AND METHODS

Two-year small-plot field experiments with grain maize were established in 2008 and 2009 in co-operation with the KWS Osiva company on a Humic Cambisol located near Miroslav in the Czech Republic (South Moravia, approx. 50 km south of Brno, 48°57'03.80''N, 16°21'55.28''E, 272 m above sea level). The mean annual temperature at the site was 10.5°C in 2008 and 10.4°C in 2009, and the corresponding mean annual precipitation was 495 and 623 mm. For the experiment we intentionally selected a plot with a very high supply of available nutrients in the soil according to Mehlich III (mg/kg): P > 185, K > 420, Ca > 5400 and Mg > 230) and an alkaline soil reaction ($pH/CaCl_2$ = 7.7). The texture class was medium-heavy and soil type was a Phaeozem (PH).

In addition to the zero level (N0), nitrogen rates of 120 (N1) and 240 (N2) kg/ha (Table 1) were selected, taking into account the vulnerable area restrictions according to the EU Directive 108/2008, where the limit for N fertilisation for both silage and grain maize is 260 kg N/ha.

In both years the grain maize hybrid KWS 2376 (FAO 340) was used after conventional pesticide treatments. The seed rate was 74,100 seeds/ha and seed drilling was carried out on 18 April in 2008 and on 20 April in 2009.

The experimental plots measured 6 m × 30 m (8 rows with 0.75 m spacing). Urea was applied manually into rows 0.1 m from the seed and harrowed into the soil to avoid N loss. Supplementary fertiliser was applied at the 5-leaf stage by scattering it manually over the soil surface. Table 1 shows the individual treatments, each of which had 4 replicates. In both years, weeds were controlled by conventional chemical spraying. During manual harvest (October 10, 2008; October 12, 2009) 15 cobs from each plot were taken in the DC 89 stage (dry straw) for sampling following yield measurements.

Grain samples were dried at 60°C to constant weight. For determination of total nitrogen content, the plant material was subjected to a liquid ashing procedure in H_2SO_4 solution (1:20 w/v) and analysed by the Kjeldahl method on a KJELTEC AUTO 1030 Analyzer (Tecator, Höganäs, Sweden).

Grain samples dried at 60°C to constant weight were hydrolysed by HCl (c = 6 mol/l) at a temperature of 115°C and used for total amino acids analysis. Sulphur-containing amino acids were determined before hydrolysis by oxidation with 15 ml HCOOH and H_2O_2 for a period of 16 h at 2°C. Chromatography analysis of hydrolysate was carried out using Na-citrate buffer and ninhydrin on an automatic amino acid analyzer AAA 400 (Ingos, Prague, Czech Republic), (Kráčmar et al. 1998).

The data were statistically analysed by means of the Statistica statistical programme using the ANOVA. The Scheffe's test ($P < 0.05$) was used to determine statistically significant differences between the treatments. Correlations were also examined using the Pearson's correlation coefficients (r).

Table 1. Design of experiment

Treatment No.	Fertiliser treatment	N dose (kg/ha)	
		before sowing	at 5-leaf stage
1	N0	0	0
2	N1	120	0
3	N2	120	120

RESULTS AND DISCUSSION

Under field conditions, N fertilisation status is one of the major factors influencing plant growth (Mengel and Kirkby 2001). A significant grain N increase with applied N dose was only observed in 2008, between treatments N0 and N1 (Table 2). Thiraporn et al. (2008) also reported enhancement of maize grain N content with increasing dose of N (from 80 to 160 kg N/ha), while Eppendorfer et al. (1985) detected an increase in total grain N from 1.06 to 2.68% N with the doses they applied. Positive correlations were found in our experiment between grain N content and the following essential amino acids: His ($r = 0.688$), Ile ($r = 0.679$), Leu ($r = 0.698$), Met ($r = 0.628$), Phe ($r = 0.723$), Val ($r = 0.656$) and the non-essential amino acids Ala ($r = 0.660$), and Glu ($r = 0.744$).

Nitrogen fertilisation can be used for nutritional improvement of the human diet by increasing and maintaining protein and essential amino acid contents (Thanapornpoonpong et al. 2008). An important nutritional limitation of maize endosperm is its amino acid balance. Deficiency in certain amino acids reduces the availability of others present in abundance. Seebauer et al. (2004) reported that N supply (0 and 168 kg N/ha) had a variable effect on individual amino acid levels in young maize cobs. However increasing N concentrations were associated with decrease in crude protein of lysine, methionine, cystine, threonine, tryptophan and, generally, with increase in isoleucine, leucine, phenylalanine and glutamate (Eppendorfer et al. 1985). Shewry (2007) reported that the nutritional quality of cereal grain also decreases with increasing grain protein content, as an increasing proportion of the nitrogen is incorporated into prolamin storage proteins.

Table 2. Content of total N in grain (% of DM)

Treatment No.	Fertiliser treatment	% N (of DM)
Year 2008		
1	N0	1.52[a]
2	N1	1.66[b]
3	N2	1.64[ab]
Year 2009		
1	N0	1.45[a]
2	N1	1.50[a]
3	N2	1.47[a]

Values for each year marked with different letters are significantly different ($P < 0.05$)

Senescence of the plant activates proteases and enables the activation of nutrients and amino acids for the production of seeds. Senescence is the highly regulated last developmental phase of plant organs and tissues, and is optimized to allow nutrient remobilization to surviving plant parts, such as seeds of annual crops. High leaf carbohydrate to nitrogen (C:N) ratios were implicated in the induction or acceleration of the senescence process (Parrott et al. 2010). During seed production the aleurone layer is formed which contains very interesting proteins, i.e. metallothionein-like proteins (Steinum et al. 1998). These proteins are also connected with oxidative stress (Baird et al. 2006) which is so typical for the late vegetation stage of the plant and metallothioneins play a role in heavy metal detoxification, especially in respect to cadmium, copper, and zinc (Hamer 1986). At the same time these proteins are cystein-rich proteins. This is again connected with the plant's demand for available amino acids which are necessary for the production of proteins forming the aleurone layer (Chittenden et al. 1978).

In terms of essential amino acids contents, there were significant differences between the treatments in 2008 except for lysine and methionine (Table 3). Mossé and Huet (1990) reported a decrease in lysine (g/16 g N) in maize grain in relation to increasing concentration of total grain nitrogen (g/100 g DM).

Lysine is synthesised through the aspartic acid metabolic pathway, which also leads to the formation of threonine, methionine and isoleucine (Azevedo and Lea 2001).

In our 2008 experiment, the highest N dose (treatment N2) significantly decreased the content of threonine, valine, iosleucine and leucine compared with the lower N dose (N1) or the unfertilised control (N0). A similar trend was identified for histidine, but its content was reduced only by N2 compared with N1.

In 2009 there were significant differences between treatments in amino acid content except for lysine, histidine and arginine (Table 3). The content of other amino acids was influenced by N fertilisation, with the highest dose of N (treatment N2) reducing the content of Thr, Val, Ile, Leu and Phe compared with the other two treatments. The trend from 2008 was thus confirmed. A possible reason for the decrease in these amino acids at the highest N dose could be a deficiency of carbon skeletons for N assimilation (Oaks 1992, Mengel and Kirkby 2001). There was a significant increase only in the content of methionine after nitrogen

Table 3. Total content of essential amino acids (EAA) in grain (mg/g DM)

Treat. No.	Fertiliser treatment	Met	Thr	Val	Ile	Leu	Phe	His	Arg	Lys
2008										
1	N0	1.88[a] ± 0.07	2.50[a] ± 0.07	3.85[a] ± 0.12	2.74[a] ± 0.11	9.83[a] ± 0.43	3.68[a] ± 0.17	2.21[ab] ± 0.06	3.73[a] ± 0.07	2.14[a] ± 0.03
2	N1	1.99[a] ± 0.05	2.47[a] ± 0.06	3.90[a] ± 0.06	2.79[a] ± 0.06	10.32[a] ± 0.19	3.82[a] ± 0.11	2.26[a] ± 0.04	3.71[a] ± 0.10	2.05[a] ± 0.05
3	N2	1.90[a] ± 0.14	2.22[b] ± 0.10	3.38[b] ± 0.11	2.47[b] ± 0.05	8.91[b] ± 0.37	3.41[b] ± 0.08	2.06[b] ± 0.09	3.66[a] ± 0.23	2.04[a] ± 0.12
2009										
1	N0	1.49[a] ± 0.06	2.14[a] ± 0.08	2.53[a] ± 0.13	1.78[a] ± 0.08	6.67[a] ± 0.34	2.89[ab] ± 0.16	1.62[a] ± 0.04	3.36[a] ± 0.17	1.99[a] ± 0.09
2	N1	1.66[bc] ± 0.07	2.16[a] ± 0.05	2.61[a] ± 0.05	1.81[a] ± 0.05	7.24[a] ± 0.41	3.05[a] ± 0.13	1.70[a] ± 0.07	3.42[a] ± 0.13	1.94[a] ± 0.05
3	N2	1.71[c] ± 0.05	1.97[b] ± 0.10	2.06[b] ± 0.085	1.36[b] ± 0.07	5.80[b] ± 0.16	2.75[b] ± 0.13	1.65[a] ± 0.06	3.45[a] ± 0.16	1.95[a] ± 0.07

Values within columns for each year marked with different letters are significantly different ($P < 0.05$)

fertilisation (treatments N1 and N2) compared with the unfertilised control (treatment N0).

The contents of most amino acids analysed corresponded in both years to the average data presented e.g. by Harrigan et al. (2009) and Ridley et al. (2004). These sources also mention higher values for leucine and valine. Harrigan et al. (2009) noted that in contrast to results from fatty acid analysis, differences in total amino acid levels appeared to be affected by environmental factors to a greater degree than genetic factors or gene × environment interaction effects.

In comparison of the same treatments between 2008 and 2009, significant differences were found among all amino acids. Alehina (1992) reported

significant changes in amino acid composition as a result of sources, rates and types of N fertilisation. Seebauer et al. (2004) found that the major amino acids in the cob were glutamine (Gln), aspartic acid (Asp), asparagine (Asn), glutamate (Glu), and alanine (Ala). In our experiments the following order was observed: Glu > Ala > Asp (Table 4).

The contents of most non-essential amino acids analysed (Table 4) corresponded in both years to the average data presented e.g. by Harrigan et al. (2009) and Ridley et al. (2004). These sources only give significantly higher values for glutamate and tyrosine.

In 2008 there were no significant differences between treatments for Cys, Glu and Gly. The

Table 4. Total content of non-essential amino acids (NEAA) in grain (mg/g DM)

Treat. No.	Fertiliser treatment	Cys	Asp	Ser	Glu	Pro	Gly	Ala	Tyr
2008									
1	N0	2.14[a] ± 0.14	5.29[a] ± 0.22	3.18[a] ± 0.09	13.93[a] ± 0.57	9.42[ac] ± 0.74	2.82[a] ± 0.06	5.93[a] ± 0.19	1.92[a] ± 0.08
2	N1	2.16[a] ± 0.10	5.21[ab] ± 0.15	3.13[a] ± 0.07	14.38[a] ± 0.30	7.38[b] ± 0.36	2.75[a] ± 0.05	6.03[a] ± 0.11	1.94[a] ± 0.10
3	N2	2.04[a] ± 0.13	4.83[b] ± 0.21	2.87[b] ± 0.17	13.83[a] ± 0.82	8.80[c] ± 0.37	2.65[a] ± 0.17	5.38[b] ± 0.32	2.84[b] ± 0.18
2009									
1	N0	2.14[a] ± 0.03	4.50[a] ± 0.17	2.73[a] ± 0.09	9.79[ab] ± 0.17	5.21[a] ± 0.09	2.38[a] ± 0.11	4.19[ab] ± 0.12	2.28[a] ± 0.08
2	N1	2.33[b] ± 0.02	4.29[a] ± 0.09	2.95[b] ± 0.07	10.47[a] ± 0.44	5.83[b] ± 0.19	2.34[a] ± 0.04	4.44[a] ± 0.14	2.36[a] ± 0.06
3	N2	2.06[c] ± 0.03	3.94[b] ± 0.17	2.84[ab] ± 0.11	9.06[b] ± 0.48	4.82[c] ± 0.24	2.27[a] ± 0.08	3.92[b] ± 0.22	2.42[a] ± 0.12

Values within columns for each year marked with different letters are significantly different ($P < 0.05$)

highest N dose (treatment N2) reduced the content of Ser and alanine compared with the other two treatments and also decreased the content of aspartic acid compared with the unfertilised control. Treatment N1 had the lowest content of proline (2008), while in 2009 this AA was significantly reduced by the highest N dose (N2) compared with the other two treatments. Proline is an extensively studied molecule in the context of plant responses to abiotic stresses (Pavlíková et al. 2008). According to Atanasova (2008), an increase in proline and alanine could serve as an indicator of an imbalance in nitrogen nutrition. The N2 dose significantly increased only the content of tyrosine compared with the other treatments. In 2009 (Table 4) there were differences between treatments except in the case of Gly and Tyr. The highest N dose applied decreased the content of most non-essential amino acids compared with the other two treatments (Cys, Asp, Pro) or compared with the lower N dose (Glu, Ala). The amino acid alanine (Ala) accumulates markedly in response to stress in plants and is especially discussed in relation to intracellular pH regulation (Pavlík et al. 2010a,b).

Aspartic acid (Asp) participates (after its decomposition) in the formation of oxalacetate, which is part of the tricarboxylic acid cycle. This cycle is very important because the assimilation of NH_4^+ into amides and amino acids requires carbon skeletons from the tricarboxylic acid cycle (Oaks 1992). The highest N dose (treatment N2) reduced the content of aspartic acid in both years and might thus have negatively influenced the activity of tricarboxylic acid cycle and caused deficiency of carbon skeletons essential for the synthesis of other amino acids. Glycine, alanine, cysteine, hydroxyproline, serine, threonine and tryptophan form pyruvate, from which is synthesised acetyl-CoA. Arginine, histidine and glutamine are substrates for the formation of α-ketoglutarate, while isoleucine, methionine and valine create succinyl-CoA, and

tyrosine and phenylalanine create fumarate. All these compounds are necessary for the tricarboxylic acid cycle (Veech 2004).

Asparagine (Asn) is the amide of aspartic acid. Inorganic N fertiliser is assimilated in plants into asparagine, which serves as an important nitrogen carrier (Ta et al. 1984) and reflects changes in N assimilation rates. Asparagine has a N:C ratio of 2:4, which makes it an efficient molecule for the storage and transport of nitrogen. It is the major compound in the xylem for transport from the roots to the leaves (Lea et al. 2007). According to Martínek et al. (2009), the asparagine content in wheat generally increases at higher N doses, e.g. they found that increasing the N rate from 0 to 180 kg/ha increased the asparagine content by about 250%. In our experiments the opposite trend was detected in aspartate content in grain maize (Table 4). However, Neuberg et al. (2010a) describe a decrease in free asparagine content in the aboveground biomass of CULTAN-treated plants and attributed this to osmotic imbalance effects in the root zone of the fertilised plants.

There were significant differences in the contents of most amino acids within the same treatments in 2008 and 2009, with the exception of Cys (treatment N0) and Cys, Ser and Lys (treatment N2).

Table 5 shows the two-year mean contents of essential (Σ EAA), non-essential (Σ NEAA) and total (Σ AA) amino acids in maize grain in both years. There were no significant differences between treatments. In addition, grain yields were affected neither by year nor by fertilisation rate. It can be assumed that in a very fertile soil with a very high supply of available macronutrients and an adequate supply of primary, easily hydrolysable organic substances, the plants were supplied with N from the process of mineralisation during the growing season. In treatments N0, N1 and N2 in 2008, the range of yields was 13.65–14.34 t/ha. In 2009 yields were similar, with a range from 13.68 to 14.18 t/ha (Lošák et al. 2010).

Table 5. Mean content (mg/g DM) of essential, non-essential and total amino acids in maize grain

AA	2008 + 2009		
	1	2	3
Σ EAA	28.56[a] ± 4.38	29.49[a] ± 4.17	26.42[a] ± 3.99
Σ NEAA	38.95[a] ± 6.15	39.04[a] ± 4.32	37.31[a] ± 6.54
Σ AA	63.97[a] ± 10.31	64.96[a] ± 8.31	60.17[a] ± 10.38

AA – amino acids; EAA – essentials amino acids; NEAA – non-essential amino acids. Values within rows are not significantly different (*P* < 0.05)

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