The effect of different nitrogen nutrition on proline and asparagine content in plant

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ABSTRACT

Mixture of plants (*Festulolium*: *Trifolium pretense* L.) was grown in the pot experiment with different forms of nitrogen nutrition**.** The fertilizers **(**ammonium sulphate or calcium nitrate or ammonium nitrate) were applied conventionally or according to the CULTAN method (Controlled Uptake Long Term Ammonium Nutrition). The absolute growth rate (AGR) and contents of free asparagine and proline in the aboveground biomass were determined. Additional nitrogen increased the dry weight of biomass and AGR of the plants treated with sidedress application in comparison with plants treated with the CULTAN method. The results suggest increased levels of free proline in CULTAN-treated plants while those of asparagine did not increase. The significance of these observations to the loss of potential yield and the relationship between methods of application is considered.

Keywords: plant metabolism; *Fabaceae*; *Poaceae*; amino acid; nitrogen assimilation

Physiological investigations have discovered that photosynthesis is closely related to the nitrogen content in leaves and reveal that this component unit is the most important mineral nutrient for plant growth. A high nitrogen supply causes a high rate of photosynthesis and consequently a faster rate of growth and greater production of biomass. Nitrogen can be available to plant roots in several different forms, including nitrate (NO_3^-), ammonium (NH_4^+), and organic forms mainly amino acids (Mengel and Kirkby 2001).

For most of the cultivated crops, nitrate is the major source of nitrogen. As the first step of the NO_3^- assimilation pathway, NO_3^- uptake by root cells is considered to be a key step of N metabolism. Once inside, NO_3^- can be redirected out of the root cell, either by extrusion in the external medium or by unloading in the xylem vessel to reach the aerial organs (Forde and Clarkson 1999).

Physiological investigations of NO_3^- uptake by roots of many different types of plants have led to the conclusion that plants developed three types of transport system to cope with the variations in NO_3^- concentrations in cultivated soils (Crawford and Glass 1998), each showing different characteristics with respect to NO_3^- inducibility or the concentration range of nitrate in the external medium ($NO_{3\,ext}^-$) at which they operate. The first system operates at low $NO_{3\,ext}^-$, in the range of 0.2mM, even if the plants have never been supplied with nitrate. This constitutive high-affinity transport system is completed by another high-affinity system which is inducible by very low $NO_{3\,ext}^-$ (Aslam et al. 1992). When the $NO_{3\,ext}^-$ reaches higher values (> 1mM), a low-affinity transport system takes place (Glass et al. 1992).

The reduction of NO_3^- takes place in roots or in leaves (Faure et al. 2001), depending on the species and on nitrogen conditions. Nitrate is first reduced to nitrite in the cytosol by nitrate reductase. Nitrite is then translocated to the chloroplast where it is reduced into ammonium by nitrite reductase.

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The third possible fate for NO_3^-, in roots as well as in leaves, is its uptake by the vacuole where it participates in the general osmoticum or serves as a reservoir to sustain the growth process when the external nitrogen supply becomes limiting (der Leij et al. 1998).

For application of ammonium form of fertilizer the system CULTAN (Controlled Uptake Long Term Ammonium Nutrition) is used in Germany (Feng et al. 1997, Sommer et al. 2002, Weber et al. 2008, Kozlovský et al. 2009). Instead of top-dressing N-fertilizers in multiple sidedress application in cereal production, CULTAN fertilization consists of injecting the entire nitrogen amount (ammonium, urea or both) in a single dose locally to the root zone (Sommer et al. 2002). In this system, plants utilized nitrogen as ammonium.

However, ammonium nutrition turns out to be stressful to many plants, leading to a reduced growth (Britto and Kronzucker 2002, Lasa et al. 2002, Cruz et al. 2006) as it lowers the cell pH. Moreover, free ammonia can readily diffuse out of the plant, which would mean a loss of a valuable resource. Ammonium is a paradoxical nutrient ion in that, although it is a major nitrogen source whose oxidation state eliminates the need for its reduction in the plant cell (Salsac et al. 1987), and although it is an intermediate in many metabolic reactions (Siecichowicz 1988), it can result in toxicity symptoms in many, if not all, plants when cultured on NH_4^+ as the exclusive N source (Fangmeier et al. 1994, Gerendas et al. 1997).

Ammonium can be toxic to plant functions and so it must be rapidly assimilated into non-toxic organic compounds. This is achieved by the GS/GOGAT cycle or pathway that is comprised of the two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT). This pathway is of crucial importance since the glutamine and glutamate produced are donors for the biosynthesis of major N-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Lea and Ireland 1999). For the GS/GOGAT cycle to work, there is a strict requirement for N metabolism to interact with C metabolism since GS activity requires energy in the form of ATP and the GOGAT uses C-skeletons and reductant in the form of 2-oxoglutarate and reduced ferredoxin or NADH, respectively (Mengel and Kirkby 2001).

If ammonia is assimilated from nitrate it cannot accumulated and transported in its original state; it must be assimilated into amino acids. Not just any amino acid is formed to transport nitrogen around the plant; a compound with a high N/C ratio, high solubility and low reactivity is needed (Siecichowicz et al. 1988). The content of amino acids is affected by nitrogen nutrition. Ammonium-grown plants often show a higher concentration of amino acids than nitrate-grown plants and higher nitrogen supply also causes an overall increase in amino acid contents (Loqué and von Wirén 2004, Atanasova 2008).

The aim of this study is a better understanding of the effect of different nitrogen nutrition to changes in the composition of free primary amino acids synthesized in plants.

MATERIALS AND METHODS

The effect of nitrogen nutrition on changes in the composition of free primary amino acids in plants was investigated in a pot experiment. For the experiment mixture of Festulolium (cv. FELINA ^{PO}; *Poaceae*, *Lolium multiflorum* Lamk. × *Festuca arundinacea* Schreber.) and *Trifolium pretense* L. (cv. Start; *Fabaceae)* was sown into plastic pots containing soil (10 kg of Chernozem; pH_{KCl} = 7.2, C_{ox} = 1.83%, CEC = 258 $mmol_{(+)}$/kg). As leaves began to form plants were treated with nitrogen (3 g per pot) in solution of ammonium sulphate – $(NH_4)_2SO_4$, or calcium nitrate $Ca(NO_3)_2$ or ammonium nitrate (NH_4NO_3) using different type of application (sidedress application-A or CULTAN method-B). For CULTAN method solution was applied into top soil (100 mm depth) at two points of pot.

The plants (6 plants of *Festulolium* and 6 plants of *Trifolium* per pot) were cultivated under natural light and temperature conditions at the experimental hall of the Czech University of Life Sciences in Prague, Czech Republic. The water regime was controlled and the soil moisture was kept at 60% MWHC.

Plants were harvested 1, 3, 5, 22, 60 and 125 days after treatment. Samples were kept frozen in liquid nitrogen for transport and then at –30°C until extraction procedure.

Samples were homogenized in liquid nitrogen and total amino acid compounds were extracted from 1 g (fresh weight) frozen plant tissue with 10 ml of methanol containing redistilled water (7:3 v/v). Homogenate was centrifuged at 9 000 g during 20 min. Supernatant was removed, filtered and the amino acids were determined using an EZ-faast amino acid analysis procedure (Phenomenex, USA). Samples were analyzed for amino acid content by the gas chromatography coupled with mass spectrometry detection using a HP 6890N/5975 instrument (Agilent Technologies, USA; Pavlík et al. 2010).

Table 1. N-content (N %) in aboveground dry biomass of plant mixture (*Festulolium*: *Trifolium pratense* L.)

Time (days)*	Calcium nitrate		Ammonium sulphate		Ammonium nitrate	
	A	B	A	B	A	B
1	3.6 ± 0.03	3.6 ± 0.03	3.8 ± 0.02	3.2 ± 0.02	3.8 ± 0.01	3.8 ± 0.02
3	3.7 ± 0.02	3.4 ± 0.02	4.0 ± 0.03	4.1 ± 0.01	4.1 ± 0.02	3.8 ± 0.03
5	4.0 ± 0.04	4.0 ± 0.01	3.4 ± 0.05	4.0 ± 0.03	4.3 ± 0.05	3.9 ± 0.02
22	2.8 ± 0.02	3.3 ± 0.03	3.2 ± 0.04	3.4 ± 0.04	3.3 ± 0.04	3.0 ± 0.03
30	2.8 ± 0.01	3.2 ± 0.02	3.1 ± 0.03	3.2 ± 0.03	3.1 ± 0.05	2.9 ± 0.05
125	2.7 ± 0.03	3.1 ± 0.04	2.8 ± 0.02	3.0 ± 0.02	3.0 ± 0.04	2.7 ± 0.03

*days after application of fertilizers; values represent the mean (n = 3) ± SD. A – sidedress application; B – CULTAN method

All gases used in this study were purchased from Messer, Inc. (Sulzbach, Germany) and were of chromatographic purity. All other chemicals and laboratory equipment were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

The dried aboveground plant biomass was used for determination of total nitrogen, ammonium and nitrate nitrogen contents. For determination of total nitrogen content the plant material was decomposed by a liquid ashing procedure in H_2SO_4 solution (1:20 w/v) and analyzed by the Kjeldahl method on a KJELTEC AUTO 1030 Analyzer (Tecator).

The significant difference among sidedress application and the CULTAN method was determined at the 95% confidence level using Tukey's honest significant difference test. Statistica for Windows version 7.0 CZ was used (StatSoft, Inc., Tulsa, OK). Values expressed as μmoles per gram fresh weight were used to perform the statistical analysis.

RESULTS AND DISCUSSION

Nitrogen content in plant decreases as the crop develops and its structure and biochemical composition change. Leaves with a large content of proteins etc. are formed in early growth, whereas supporting stems with more cellulose and lignin and little protein are formed later (Lawlor et al. 2001). Percentage contents of nitrogen in dry biomass in this experiment were comparable at all harvesting periods (Table 1). No significant differences were found between treatments. However due to their time of distribution the concentrations of nitrogen in the aboveground biomass were slightly different. Nitrogen accumulation was highly related to plant growth rate and to biomass accumulation.

Both treatments (A and B) of plant mixture (*Festulolium*: *Trifolium pratense* L.) showed very different responses to ammonium and nitrate nutrition (Table 2).

Both treatments were grown in the presence of 3 g N per pot supplied as ammonium sulphate, calcium nitrate or ammonium nitrate. Plants treated with local application of nitrogen markedly stopped growth immediately after injection of nitrogen fertilizers. Pavlík et al. (2010) confirmed yield decrease after nitrogen CULTAN application. The yield decrease of maize aboveground biomass treated with the CULTAN system compared to conventional application varied between 22–37% in the first two sampling periods. Difference in harvest period was only 7–9%.

The inhibitory effect was even more dramatic straight in the place of treatment by ammonium

Table 2. The yield (g/pot) of plant mixture (*Festulolium*: *Trifolium pratense* L.)

Calcium nitrate		Ammonium sulphate		Ammonium nitrate	
A	B	A	B	A	B
549.6	424.5	592.3	516.3	634.8	607.2

Values represent total grams of fresh weight per pot during six harvesting time

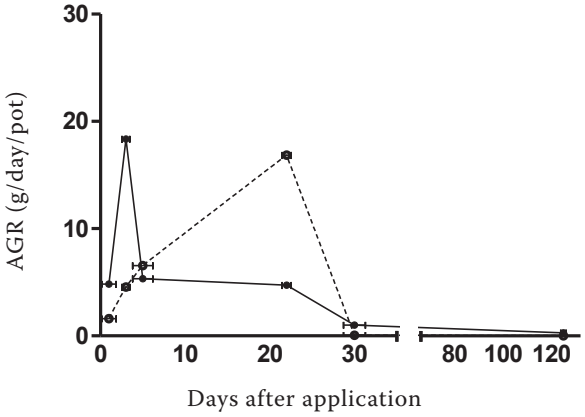

Figure 1. Mixture of *Festulolium*: *Trifolium pratense* L. absolute growth rate (AGR) response to calcium nitrate applied conventionally (straight line) and using CULTAN method (dashed line). Every point is the mean from three replications and the bars are ± SD

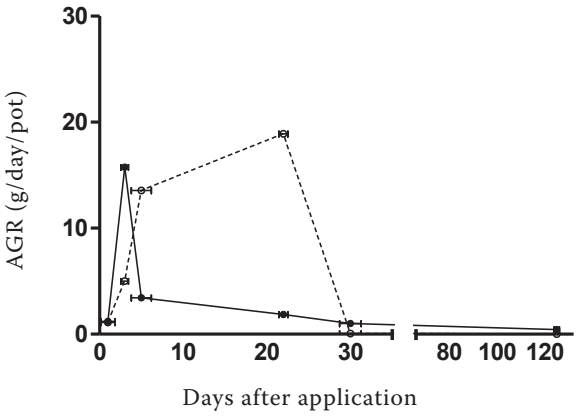

Figure 2. Mixture of *Festulolium*: *Trifolium pratense* L. absolute growth rate (AGR) response to ammonium sulphate applied conventionally (straight line) and using CULTAN method (dashed line). Every point is the mean from three replications and the bars are ± SD

sulphate or ammonium nitrate: leaves showing visible signs of senescence such as wilting, brown spots and occasionally a beginning detachment from the stem. Biomass reduction caused by using the CULTAN method was approximately 23% (calcium nitrate), 13% (ammonium sulphate) and 5% (ammonium nitrate) of weight in comparison with plants treated with conventional application. However Cruz et al. (1993) and Cramer and Lewis (1993) found lower plant biomass accumulation with ammonium than with nitrate nutrition.

Nitrogen fertilizer treatments affected biomass accumulation during the whole season measured as absolute growth rate (AGR). The absolute growth rate (AGR) as a function of added nitrogen and type of application varied considerably (Figures 1–3). The AGR was affected by the type of fertilizer and application during all plant growth periods in this pot experiment. During the first growth period from transplanting to flowering, onset sidedress application of nitrogen doses increased AGR in comparison with the CULTAN method. From flowering onset to first mature pods the AGR was reduced by sidedress application. Conversely, the CULTAN method had a positive effect on AGR in the last development period from first mature pods to leaf senescence. However, this apparent beneficial effect of the CULTAN method on AGR late in the plant life cycle may be related to the fact that CULTAN-stressed plants delayed their early growth and development and subsequently continued growing longer into the season resulting in a higher AGR, while non-stressed plants had already reached leaf senescence.

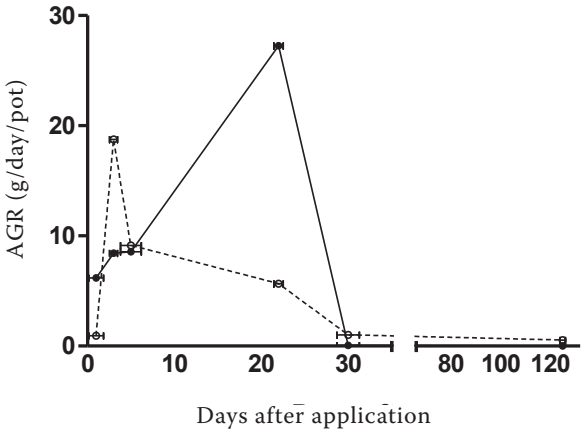

Figure 3. Mixture of *Festulolium*: *Trifolium pretense* L. absolute growth rate response to ammonium nitrate applied conventionally (straight line) and using CULTAN method (dashed line). Every point is the mean from three replications and the bars are ± SD

Free amino acid (AA) concentrations (especially of proline and asparagine) were analyzed in the aboveground biomass of mixture (*Festulolium*: *Trifolium pretense* L.) plants (Table 3–4). The total amino acids content significantly changed as a result of fertilization. When $Ca(NO_3)_2$ was applied conventionally the average content of total AA was the highest, 1120.8 µmol/g fresh mass (FM), in comparison with the result of the CULTAN method, 518.7 µmol/g FM. When $(NH_4)_2SO_4$ and NH_4NO_3 were used as nitrogen sources, the content of total amino acids decreased with the CULTAN

Table 3. Asparagine content in aboveground dry biomass of plant mixture (*Festulolium*: *Trifolium pretense* L.)

Time (days)*	Calcium nitrate		Ammonium sulphate		Ammonium nitrate	
	A	B	A	B	A	B
1	84.0 ± 2.4	122.9 ± 7.6	23.8 ± 1.3	18.6 ± 0.8	113.3 ± 2.4	38.8 ± 1.5
3	437.5 ± 8.6	217.1 ± 5.2	23.4 ± 2.2	52.6 ± 1.1	435.8 ± 15.2	363.7 ± 12.9
5	347.2 ± 6.4	242.7 ± 10.3	128.9 ± 5.6	50.7 ± 2.1	424.1 ± 12.4	274.5 ± 11.3
22	340.6 ± 15.2	230.7 ± 12.3	110.7 ± 6.4	48.2 ± 1.3	400.5 ± 11.5	270.2 ± 9.8
30	300.6 ± 9.9	240.6 ± 10.6	90.5 ± 3.6	45.3 ± 1.5	350.3 ± 9.6	240.3 ± 8.7
125	248.2 ± 15.7	180.6 ± 12.5	80.4 ± 2.6	40.2 ± 1.2	384.2 ± 12.4	144.3 ± 3.5

*days after application of fertilizers; values in μmol/g FM (fresh matter) represent the mean (n = 3) ± SD. A – sidedress application; B – CULTAN method

method to 384.2 and 753.4 μmol/g FM in comparison with the values of sidedress application, 666.2 and 914.5 μmol/g FM, respectively. According to Alehina (1992) significant changes in amino acid composition were observed as a result of sources, rates and types of nitrogen fertilization. According to Ueda et al. (2008) ammonium supply strongly affected the content and synthesis of the amino acids in plants. The supply of ammonium increased considerably the concentrations of the primary amino acids; asparagine was the most predominant acid, followed by glutamine.

Supplied inorganic nitrogen is assimilated in plants into asparagine, which serves as important nitrogen carriers (Ta et al. 1984) and reflects changes in the nitrogen 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 root to the leaves. In nitrogen-fixing plants, asparagine is the major amino acid in all plant parts and could account for 60–80% of the total amino acids in nodulated roots, leaves and fruits (Lea et al. 2007).

Our results showed that levels of free asparagine were significantly higher in plants treated with sidedress application (A) (Table 3). Moreover, this marked increase of free asparagine in comparison with the CULTAN method (B) occurs in all types of nitrogen sources (calcium nitrate, ammonium sulphate and ammonium nitrate). The highest concentration of free asparagine was found in plants fertilized by calcium nitrate and ammonium nitrate as a nitrogen source. Weber et al. (2008) reported that application of different N fertilizers affected the concentration of free asparagine in plant. According to Martínek et al. (2009) asparagine content in wheat was generally increasing at higher nitrogen doses and nitrogen dose increase from 0 to 180 kg/ha increased the asparagine content to about 250%.

On the other hand, a decrease of free asparagine content in the aboveground biomass of CULTAN-

Table 4. Proline content in aboveground dry biomass of mixture (*Festulolium*: *Trifolium pretense* L.)

Time (days)*	Calcium nitrate		Ammonium sulphate		Ammonium nitrate	
	A	B	A	B	A	B
1	2.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	2.3 ± 0.1	1.6 ± 0.1	0.8 ± 0.1
3	30.0 ± 1.3	30.5 ± 1.2	6.2 ± 0.1	15.9 ± 0.8	7.5 ± 0.1	52.3 ± 2.1
5	17.0 ± 1.1	90.6 ± 2.3	22.0 ± 1.0	96.3 ± 3.3	9.4 ± 0.1	30.6 ± 1.2
22	16.1 ± 1.0	17.1 ± 1.3	21.7 ± 2.3	24.1 ± 1.9	1.6 ± 0.1	25.2 ± 0.6
30	15.6 ± 0.9	16.6 ± 2.2	20.2 ± 1.2	25.6 ± 2.2	1.0 ± 0.1	1.0 ± 0.1
125	14.5 ± 0.3	15.8 ± 1.8	14.3 ± 0.8	8.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1

*days after application of fertilizers; values in μmol/g FM (fresh matter) represent the mean (n = 3) ± SD. A – sidedress application; B – CULTAN method

treated plants was detected. It can be due to the osmotic disbalance effects in root zone of fertilized plants. This hypothesis also confirmed an increase of free proline (Table 4) in CULTAN-treated plants.

Proline is an extensively studied molecule in the context of plant responses to abiotic stresses (Pavlíková et al. 2008). A marked increase of free proline content in aboveground biomass was detected in our experiment. Major elevation of its concentration occurs on 3rd and 5th day after application of fertilizer in CULTAN-treated plants in comparison with plants treated with sidedress application. The levels of free proline ranged in dependence on application method and nitrogen source: 6.2–30 μmol/g FM in plants fertilized conventionally in comparison to 30.6–96.3 μmol/g FM in the CULTAN-treated plants. The results confirmed that free proline accumulated after nitrogen application with the CULTAN method. According to Neuberg et al. (2010) the significant increase of proline concentration was observed in plant aboveground biomass of red clover under injection treatment in contrast to sidedress application. Many plants accumulate this compatible solute under water deficit (Aspinall and Paleg 1981), salinity (Ashraf and Harris 2004), low temperature (Naidu et al. 1991), high temperature, and some other environmental stresses. According to Atanasova (2008) the increase of proline and alanine could serve as an indicator for unbalanced nitrogen nutrition.

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