DEPENDENCE OF FREE ALANINE LEVELS IN *FESTULOLIUM* AND *TRIFOLIUM PRATENSE* L. ON TYPES OF N NUTRITION*

M. Neuberg, D. Pavlíková, J. Balík

Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Agro-Environmental Chemistry and Plant Nutrition, Prague, Czech Republic

This study aims to reveal and to compare effects of two various systems of nitrogen (N) nutrition, namely (1) injection by CULTAN (Controlled Uptake Long Term Ammonium Nutrition) method and (2) conventional application, on free alanine, total N content and visual symptoms in *Festulolium* and *Trifolium pratense* L. plants. Calcium nitrate (CN) or ammonium sulphate (AS) were used as N source in the pot experiment. The yield of *Festulolium* and *Trifolium pratense* L. above-ground biomass was more substantially enhanced after conventional application of both AS or CN in comparison with CULTAN method. The highest concentration of free alanine was detected in conventional-treated *Festulolium* with AS and in conventional-treated clover with AS. The contents of N in the above-ground dry biomass were comparable at all harvesting periods. Visual symptoms suggest that N applied conventionally can maintain better distribution patterns of nutrients and provide improved conditions for growth and N uptake.

nitrogen nutrition; free alanine; *Festulolium*; clover

INTRODUCTION

Festulolium (Poaceae), a cross between *Lolium multiflorum* Lamk. × *Festuca arundinacea* Schreber., and clover (Fabaceae, *Trifolium pratense* L.) are commonly used as field crops with high request for N. They obtain N from soil, where is usually available to plants in an inorganic form such as nitrate (NO_3^-), nitrite (NO_2^-) or ammonia (NH_4^+) (Sakakibara et al., 2010), arising from mineralization of organic matter or fertilizers.

Subsequent use of N by plants involves several steps including uptake, assimilation, translocation and, when the plant is ageing, recycling and remobilization (Masclaux-Daubresse et al., 2010). There are, however, complex interactions with many other aspects of N metabolism, including (1) storage and remobilization of nitrate in different parts of the plant, (2) *de novo* ammonium assimilation, (3) recycling of ammonium released during photorespiration (Hirel, Lea, 2001), (4) distribution of N between the highly branched pathways of amino acid biosynthesis (Morot-Gaudry et al., 2001) and (5) multifarious fates of free amino acids, which can be exported, stored in the vacuole, used for protein synthesis or diverted into secondary metabolic pathways leading to phenylpropanoids, alkaloids and tetrapyroles (Heldt, 1996).

At the conventional surface application of nitrogen fertilizers, the plants, both grass and clover, take up nitrogen mainly in the nitrate form. At the same time, clover, similar to other legumes fertilized with mineral nitrogen, produces nodules as a prerequisite of N_2 fixation. Nodule formation and therefore N_2 fixation may be reduced when N is supplied as mineral fertilizer (Kozlovský et al., 2009).

Nitrogen fertilizers can be applied by conventional application or by an alternative method. An alternative method of fertilization is the CULTAN (Controlled Uptake Long Term Ammonium Nutrition) method; it uses a special injection technique to apply nitrogen solutions rich in ammonium ions into soil, into a depth of 7–20 cm, where it is retained in the spots with high concentration of ammonia, the so-called depots (Sommer 2002; Kozlovský et al., 2009). Although, it is well known that high concentrations of NH_4^+ can be toxic to plants leading to severe growth depression and physiological stresses (Britto, Kronzucker, 2002).

Amino acid metabolism has the central role in stress resistance of plants. Changes in the content of free amino acids in Poaceae have been studied for various

* Supported by the Ministry of Agriculture of the Czech Republic, Project No. QH71077, and by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046070901.

types of stresses. Acidity stress increased the proline content and reduced the histidine and arginine content in *Pennisetum clandestinum* Hochst. (Sidari et al., 2004). The contents of glutamine, asparagine and arginine in *Deschampsia xexuosa* (L.) Trin. increased at nitrogen stress (Ohlson et al., 1995). The content of proline has been observed to accumulate in *Hordeum distichum* L. and *Triticum aestivum* L. after cold stress (Chu et al., 1999) and accumulation of alanine under anaerobic conditions in *Hordeum vulgare* roots were observed (Good, Muench, 1992).

In the present study, we would like to address a question whether free alanine accumulation in the *Festulolium* and clover can be manipulated by an addition of N, either in form of ammonium sulphate (AS) or calcium nitrate (CN), by (1) a newly developed CULTAN method or (2) conventional application and to correlate the biochemical effects with the physiological effects, expressed as visual symptoms and biomass reduction, at time intervals after exposure.

MATERIALS AND METHODS

Experimental setting

Festulolium and clover seeds were germinated and plants were cultivated in pots (containing 10 kg of chernozem – Suchdol; from May to September) in the outside vegetation hall (Czech University of Life Sciences Prague, Czech Republic) under natural light and temperature conditions (long-term mean for selected area). The water regime was controlled and the soil moisture was kept at 60% MWHC (maximum water holding capacity).

The experimental setup consisted of ammonium sulphate [$(NH_4)_2SO_4$] or calcium nitrate [$Ca(NO_3)_2$] treatments using either (1) conventional application or (2) CULTAN method (i.e. injecting N application; Feng et al., 1997; Sommer et al., 2002; Kozlovský et al., 2009). N fertiliser was applied to the developing plants after leaves began forming, namely at rates 40 ml of 2.68 M AS or CN (equivalent of 3 g of N per pot by conventional application or into top soil, 100 mm depth, on two points of pot by CULTAN method).

Plants (above-ground biomass) were harvested 1 (1), 3 (2), 5 (3), 22 (4), 60 (5) and 125 (6) days after treatment. Samples were frozen in liquid nitrogen for transport and stored at –30°C until next extraction procedure.

Determination of nitrogen and nitrate

The dried above-ground biomass was used for determination of total N contents. For determination of total N 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).

Extraction of amino acids

Samples were homogenized in liquid nitrogen and total amino acid compounds were extracted from 1 g (fresh weight; FW) above-ground frozen plant tissue with 10 ml of absolute methanol containing redistilled water (7:3 v/v). Homogenate was centrifuged at 9000 g during 20 min. Supernatant was removed, filtered and EZ: fast procedure was made.

Samples were analyzed for amino acid contents by the gas chromatography coupled with mass spectrometry detection using a HP 6890N/5975 instrument (Agilent Technologies, USA; Pavlík et al., 2010a).

Statistical analysis

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

Table 1. Nitrogen contents in above-ground dry biomass (DW) of *Festulolium* and clover

Time of harvesting	*Festulolium*				Clover			
	CN		AS		CN		AS	
	1	2	1	2	1	2	1	2
1	2.99	1.55	2.13	1.98	2.98	3.92	3.45	3.73
2	3.00	2.74	2.41	2.31	3.76	4.09	4.16	4.03
3	3.36	3.24	2.85	3.24	3.90	4.08	3.84	4.12
4	4.23	3.81	4.35	4.47	3.32	3.49	3.19	2.81
5	3.78	3.65	3.85	3.63	3.19	3.36	3.18	2.54
6	3.45	3.47	3.52	3.52	3.14	3.15	3.19	2.36

Values represent percentage content of N (%; 1 – conventional application; 2 – local application; CN – calcium nitrate; AS – ammonium sulphate)

RESULTS

Biomass accumulation

Various fertilizing systems and their relationship to N (NH_4^+ or NO_3^-) nutrition were assessed in the present study to follow the dynamics of N uptake; namely (1) a conventional application and (2) a local application by CULTAN method.

Both treatments of *Festulolium* showed very different responses to ammonium or nitrate nutrition in dependence on type of N application. The yield of above-ground biomass was higher after conventional application of both AS or CN (167.5 g FW and 171.4 g FW per pot, respectively) in comparison with CULTAN method (140.3 g FW and 141.4 g FW, respectively). The same was found for clover biomass (data not shown).

Above-ground plant N

The contents of N in the above-ground dry biomass were comparable at all harvesting periods although slight differences between individual variants, especially due to the time of distribution and soil properties, were found. For all treatments, the contents of N increased markedly between the start of vegetation and flowering and then tended to decrease slightly or to remain constant up to maturity (Table 1).

Free alanine in above-ground biomass

Amino acid metabolism has the central role in abiotic stress resistance of plants. Alanine is specially discussed in relation to intracellular pH regulation. Our results confirmed changes of free alanine levels in plant growing under ammonium nitrogen stress. The free alanine content significantly changed as a result of N nutrition (Figs. 1–4). The highest concentration (55.9 µmol.g^{-1} FW; 54.6 µmol.g^{-1} FW) of free alanine was detected in conventional-treated *Festulolium* with AS and in conventional-treated clover with AS. The levels of free alanine ranged in dependence on the type of application and nitrogen source; from 12.3 to 21.6 µmol.g^{-1} FW in plants fertilized by conventional application while from 16.2 to 66.1 µmol. g^{-1} FW in CULTAN-treated plants.

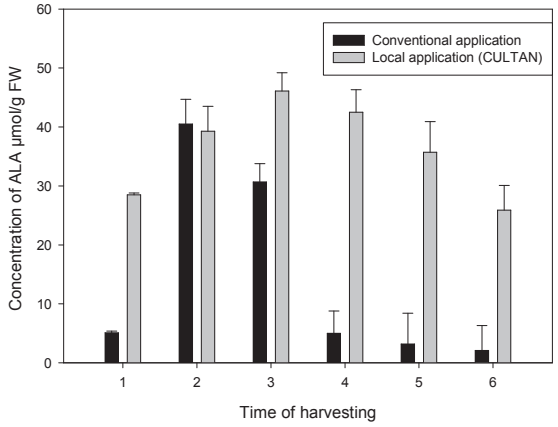

Fig. 1. Endogenous levels of free alanine in Festulolium above-ground biomass during a 125 days period after application of calcium nitrate. Calcium nitrate (CN) was applied by conventional application and CULTAN method

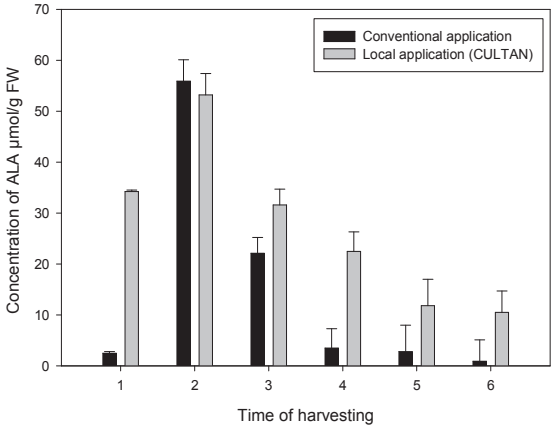

Fig. 2. Endogenous levels of free alanine in Festulolium above-ground biomass during a 125 days period after application of ammonium sulphate. Ammonium sulphate (AS) was applied by conventional application and CULTAN method

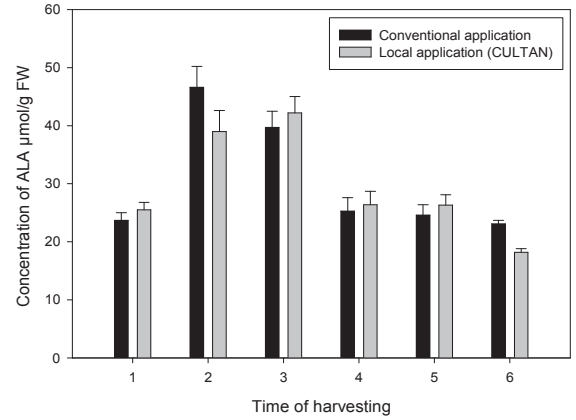

Fig. 3. Endogenous levels of free alanine in clover above-ground biomass during a 125 days period after application of calcium nitrate. Calcium nitrate (CN) was applied by conventional application and CULTAN method

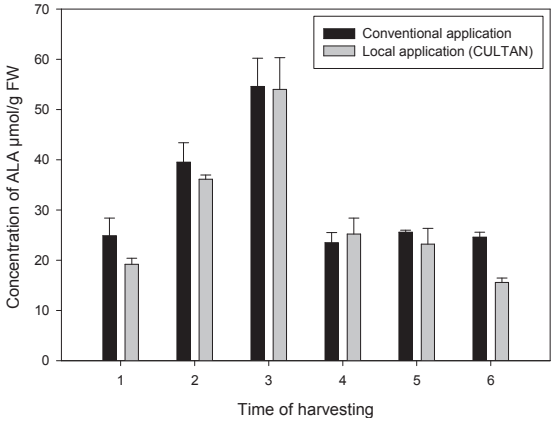

Fig. 4. Endogenous levels of free alanine in clover above-ground biomass during a 125 days period after application of ammonium sulphate. Ammonium sulphate (AS) was applied by conventional application and CULTAN method

DISCUSSION

The dynamics of N uptake with regard to free alanine levels in *Festulolium* and clover plants were observed using various forms of N nutrition (NH_4^+ or NO_3^-) and different types of N application.

Biomass accumulation

In our work, *Trifolium* showed signs of higher sensitivity to NH_4^+nutrition compared to *Festulolium* plants. According to Gerendas et al. (1997) the chlorosis of leaves, and the overall suppression of growth were found (both in *Festulolium* and clover). Yield depressions among sensitive species range from 15% to 60% (Chaillou et al., 1986) and can even lead to death (de Graaf et al., 1998). Plants treated by locally applied N markedly reduced growth immediately after injection of AS or CN. The inhibitory effect was more dramatic directly at the place of treatment by AS after which the leaves showed visible signs of senescence such as wilting, brown spots, and occasionally a beginning detachment from the stem, for both plants. These results are compatible with the significant reduction of the wheat dry matter production in shoots and roots (Garnica et al., 2010).

Higher differences in biomass accumulation were found by using CULTAN method, where a significant reduction of biomass weight was detected in comparison with plants treated by conventional application. The similar results were published by Neuberg et al. (2010, 2011).

Above-ground plant N

The amount of plant N increased markedly from the start of vegetation to early flowering phase in *Festulolium* and *Trifolium pratense* L., and then tended to remain constant up to maturity. This suggestion is in agreement with the earlier results by Lawlor et al. (2001) demonstrating that plants N content unit biomass (N% in dry matter) is not a fixed value and decreases as the crop develops and its structure and biochemical composition change.

Free alanine (Ala) concentrations in above-ground plant biomass

Free amino acid concentrations, especially those of alanine, were analyzed in the above- ground biomass of *Festulolium* and *Trifolium pratense* L. plants. Amino acid metabolism may play an important role in plant stress resistance, by osmotic adjustment and the accumulation of compatible osmolytes; detoxification of active oxygen species and intracellular pH regulation (Singh, 1999). According to Alehina (1992), significant changes in amino acid composition were observed as a result of sources, rates and types of nitrogen fertilization. Ueda et al. (2008) reported that ammonium supply strongly affected the content and synthesis of the amino acids in the plants.

Marked increase of free alanine content in above-ground biomass was detected in our experiment. The results showed that free alanine accumulation was higher when using the CULTAN method compared to the conventional application. Many plants accumulate alanine under anaerobic conditions and pests damages (Good, Muench, 1992).

The alanine is markedly accumulated in response to stress in plants and it is especially discussed in relation to intracellular pH regulation. Naidu et al. (1991) observed in *Triticum aestivum* L. an accumulation of alanine after exposure to low temperatures and this was explained by an accumulation of citric acid enzymes leading to an increased synthesis of the citric acid related alanine, and it was explained by a reduced entry of amino acids into the respiratory chain, and reduced rate of protein synthesis. Accumulation of alanine has also been reported in *Z. mays* L. treated with the aryloxyphenoxypropionic herbicide and it was suggested to be caused by increased synthesis of alanine and a concentrating effect due to decreased cell expansion or elongation (Snipes et al., 1987). According to Hjorth et al. (2006) the increased content of free alanine might be caused by a reduction in the rate of protein syntheses and an increased synthesis of alanine due to disturbance of the alanine aminotransferase reactions. According to Atanasova (2008) the increase of proline and alanine could serve as an indicator for unbalanced nitrogen nutrition. Similar trend was found in mixture of plants *Festulolium* and *Trifolium pratense* L. (Neuberg et al., 2010) or in maize (Pavlík et al., 2010a,b).

CONCLUSION

In this article, we have focused on the comparison of different N fertilizers (AS and CN) and types of application (conventional and CULTAN methods) on free alanine and total N content of *Festulolium* and *Trifolium pratense* L. plants. To summarize, the conventional application of nitrogen fertilizer resulted in higher above-ground biomass accumulation (for both CN or AS) as compared with CULTAN-method application. The enhanced content of free amino and free alanine support our hypothesis and suggest that N applied conventionally can maintain better distribution patterns of nutrients and provide improved conditions for growth and nitrogen uptake.

REFERENCES

ALEHINA, N.D.: Nitrogen assimilation in roots and leaves: specificity and dependence on environmental conditions. Physiology and Biochemistry of Cultural Plants, *24*, 1992: 338–343.

ATANASOVA, E.: Effect of nitrogen sources on the nitrogenous forms and accumulation of amino acid in head cabbage. Plant, Soil and Environment, *54*, 2008: 66–71.

BRITTO, D.T. – KRONZUCKER, H.J.: NH_4^+ toxicity in higher plants: a critical review. Journal of Plant Physiology, *159*, 2002: 567–584.

CHAILLOU, S. – MOROT-GAUDRY, J.F. – SALSAC, L. – LESAINT, C. – JOLIVET, E.: Compared effects of NO_3^-or NH_4^+on growth and metabolism of French bean. Physiologie Vegetale, *24*, 1986: 679–687.

CHU, T.M. – JUSAITIS, M. – ASPINALL, D. – PALEG, L.G.: Accumulation of free proline at low temperatures. Physiology, *155*, 1999: 310–317.

DE GRAAF, M.C.C. – BOBBINK, R. – VERBEEK, P.J.M. – ROELOFS, J.G.M.: Differential effects of ammonium and nitrate on three heathland species. Plant Ecology, *135*, 1998: 185–196.

FENG, K. – YAN, F. – SCHUBERT, S.: Response of *Zea mays* and *Vicia faba* to CULTAN fertilization. Zeitschrift für Pflanzenernährung und Bodenkunde, *160*, 1997: 291–293.

GARNICA, M. – HOUDUSSE, F. – ZAMARRENO, M.A. – GARCIA-MINA, M.J.: The signal effect of nitrate supply enhances active forms of cytokinins and indole acetic content and reduces abscisic acid in wheat plants grown with ammonium. Journal of Plant Physiology, *167*, 2010: 1264–1272.

GERENDAS, J. – ZHU, Z. – BENDIXEN, R. – RATCLIFFE, R.G. – SATTELMACHER, B.: Physiological and biochemical processes related to ammonium toxicity in higher plants. Journal of Plant Nutrition and Soil Science, *160*, 1997: 239–251.

GOOD, A.G. – MUENCH, D.G.: Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. Plant Physiology, *99*, 1992: 1520–1525.

HELDT, H.W.: Pflanzenbiochemie (Plant Biochemistry). Spektrum Akademischer Verlag, Heidelberg, 1996. (in German)

HIREL, B. – LEA, P.: Ammonia assimilation. In: Lea, P.J., Morot-Gaudry, J.F., eds. Plant nitrogen. Springer-Verlag, Berlin, Germany, 2001, pp. 79–100.

HJORTH, M. – MATHIASSEN, S.K. – KUDSK, P. – RAVN, H.W.: Amino acids in loose silky-bent (*Apera spica-venti* (L.) Beauv.) responding to prosulfocarb exposure and the correlation with physiological effects. Pesticide Biochemistry and Physiology, *86*, 2006: 138–145.

KOZLOVSKÝ, O. – BALÍK, J. – ČERNÝ, J. – KULHÁNEK, M. – KOS, M. – PRÁŠILOVÁ, O.: Influence of nitrogen fertilizer injection (CULTAN) on yield, yield components formation and quality of winter wheat grain. Plant, Soil and Environment, *55*, 2009: 536–543.

LAWLOR, D.W. – LEMAIRE, G. – GASTAL, F.: Nitrogen, plant growth and crop yield. In: LEA, P.J. – MOROT-GAUDRY, J.F., eds.: Plant Nitrogen. Springer-Verlag, Berlin, Germany, 2001, pp. 343–367.

MASCLAUX-DAUBRESSE, C. – DANIELE-VEDELE, F. – DECHORGNAT, J. – CHARDON, F. – GAUFICHON, L. – SUZUKI, A.: Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany, *105*, 2010: 1141–1157.

MOROT-GAUDRY, J.F. – JOB, D. – LEA, P.J.: Amino acid metabolism. In: Lea, P.J., Morot-Gaudry, J.F., eds.: Plant Nitrogen. Springer-Verlag, Berlin, Germany, 2001, pp. 167–212.

NAIDU, B.P. – PALEG, L.G. – ASPINALL, D. – JENNINGS, A.C. – JONES, G.P.: Amino acid and glycine betaine accumulation in cold-stressed wheat seedlings. Phytochemistry, *30*, 1991: 407–409.

NEUBERG, M. – PAVLÍKOVÁ, D. – PAVLÍK, M. – BALÍK, J.: The effect of different nitrogen nutrition on proline and asparagine content in plant. Plant, Soil and Environment, *56*, 2010: 305–311.

NEUBERG, M. – PAVLÍKOVÁ, D. – ŽIŽKOVÁ, E. – MOTYKA, V. – PAVLÍK, M.: Different types of N nutrition and their impact on endogenous cytokinin levels in *Festulolium* and *Trifolium pratense* L.. Plant, Soil and Environment, *57*, 2011: 381–387.

OHLSON, M., – NORDIN, A. – NASHOLM, T.: Accumulation of amino acids in forest plants in relation to ecological amplitude and nitrogen supply. Functional Ekology, *9*, 1995: 596–605.

PAVLÍK, M. – PAVLÍKOVÁ, D. – BALÍK, J. – NEUBERG, M.: The contents of amino acids and sterols in maize plants growing under different nitrogen conditions. Plant, Soil and Environment, *56*, 2010a: 125–132.

PAVLÍK, M. – PAVLÍKOVÁ, D. – VAŠÍČKOVÁ, S.: Infrared spectroscopy-based metabolomic analysis of maize growing under different nitrogen nutrition. Plant, Soil and Environment, *56*, 2010b: 541–548.

SAKAKIBARA, H. – TAKEI, K. – HIROSE, N.: Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science, *9*, 2010: 440–448.

SIDARI, M. – PANUCCIO, M.R. – MUSCOLO, A.: Infuence of acidity on growth and biochemistry of *Pennisetum clandestinum*. Biologia Plantarum, *48*, 2004: 133–136.

SINGH, B.K. (ed.): Biochemistry and Biotechnology. M. Dekker Inc., New York, USA, 1999, 621 p.

SNIPES, C.E. – STREET, J.E. – LUTHE, D.S.: Physiological influences of fenoxaprop on corn (*Zea mays*). Pesticide Biochemistry and Physiology, *28*, 1987: 333–340.

SOMMER, K. – SCHERER, H.W. – KUNERT, A.: CULTAN-Verfahren bei Mais (Methods for maize), Mais, 2002: 20–23. (in German)

UEDA, S. – IKEDA, M. – YAMAKAWA, T.: Provision of carbon skeletons for amide synthesis in non-nodulated soybean and pea roots in response to the source of nitrogen supply. Soil Science and Plant Nutrition, *54*, 2008: 732–737.

Received for publication on May 30, 2011
Accepted for publication on November 9, 2011

NEUBERG, M. – PAVLÍKOVÁ, D. – BALÍK, J. (Česká zemědělská univerzita v Praze, Fakulta agrobiologie, potravinových a přírodních zdrojů, Praha, Česká republika)

Závislost hladin volného alaninu u Festulolia a jetele na typu N výživy

Scientia Agric. Bohem., *42*, 2011, 147–152

Tato studie si klade za cíl porovnat účinky dvou různých systémů N výživy, a to (1) metodou CULTAN (Controlled Uptake Long Term Ammonium Nutrition) a (2) konvenční aplikace, a jejich vliv na hladiny volného alaninu a obsah celkového N u rostlin *Festulolium* a *Trifolium pratense* L. Pro tento pokus byl založen nádobový pokus, kde byl N aplikován ve formě dusičnanu vápenatého a síranu amonného. Výnos nadzemní biomasy byl vyšší, u obou rostlin, při aplikaci obou forem N konvenční metodou v porovnání s metodou CULTAN. Nejvyšší obsahy volného alaninu byly detekovány u rostlin ošetřených síranem amonným aplikovaným konvenčně. Celkové obsahy N byly srovnatelné v průběhu vegetační sezóny u jednotlivých způsobů aplikace různých forem N výživy. Vizuální symptomy naznačují vhodnější vzorec příjmu N aplikovaného konvenčně.

dusíkatá výživa; volný alanin; *Festulolium*; jetel

Contact Address:

Mgr. Marek N e u b e r g , Ph.D., Česká zemědělská univerzita v Praze, Fakulta agrobiologie, potravinových a přírodních zdrojů, Kamýcká 129, 165 21 Praha 6-Suchdol, Česká republika, tel: +420 224 382 735; e-mail: euronymos666@seznam.cz