Different types of N nutrition and their impact on endogenous cytokinin levels in *Festulolium* and *Trifolium pratense* L.

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ABSTRACT

This study aims to reveal and to compare effects of two different systems of nitrogen (N) nutrition (sidedress application or injection application) on toxicity of NH_4^+ and mixed nutrition. We investigated whether NH_4^+ or mixed (NH_4NO_3) application causes significant changes in the endogenous levels of cytokinins (CK), whole plant N and their effects on yield of selected plants. Ammonium sulphate or ammonium nitrate were used as N source in the pot experiment. The yield of *Festulolium* and *Trifolium pratense* L. above-ground biomass and roots was more substantially enhanced after sidedress application of both ammonium sources in comparison with injection application. Our results confirmed that the accumulation of CKs in plants is in correlation with their N content (R^2 = 0.66–0.98). Proportions between individual CK forms remained relatively steady and their dynamics exhibited similar trends after N application. Our results indicate that the negative effect of the application of NH_4^+ on the growth of *Festulolium* and clover plants could be effectively modulated by the presence of NO_3^-.

Keywords: poaceae; fabaceae; N nutrition; injection application; Trans-zeatin; Dihydrozeatin; conjugates of cytokinins; nitrogen uptake

It is well known that high concentrations of NH_4^+ can be toxic to plants leading to severe growth depression (Britto et al. 2001, Britto and Kronzucker 2002). Various hypotheses were put forward, aimed at identifying the cause of NH_4^+ toxicity. The majority of these hypotheses deal with the physiological changes associated with NH_4^+ assimilation and ion imbalances resulting from decreased uptake of essential cations such as K^+, Mg^{2+}, and Ca^{2+} (Roosta and Schjoerring 2007). However, numerous studies showed that the presence of NO_3^- in the nutrient solution corrected the negative effects associated with NH_4^+ nutrition in certain plant species (Houdusse et al. 2007). This beneficial effect of NO_3^- on NH_4^+ nutrition might be related to changes in the physiological pH (Babourina et al. 2007), the maintenance of appropriate carboxylate levels (Feng et al. 1998), or to specific changes in the levels of certain plant hormones and phytoregulators (Rahayu et al. 2005). In addition, NH_4^+ toxicity was associated with hormonal imbalances (Walch-Liu et al. 2000).

Phytohormones act as signals that can stimulate or inhibit growth or regulate developmental programs in plants (Fosket 1994). Various hormonal factors were implicated in the regulation of plant growth responses to environmental stimuli such as drought, salinity, soil compaction or nutritional deficiencies (Hartung et al. 1999). Several authors reported that the concentration and the form of N sources have important influence on endogenous cytokinin (CK) synthesis (Wagner and Beck 1993).

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According to some research, plants grown with the presence of NH_4^+ in the nutrient solution contain higher levels of CKs than NO_3^--fed plants (Chen et al. 1998). On the other hand, others observed that NH_4^+ nutrition induced inhibition of shoot growth and was correlated with a sharp decline in CK concentrations (Rahayu et al. 2001). Moreover, there are several reports suggesting that the accumulation of CKs is closely correlated with the nitrogen content of the plants, such as *Urtica dioica* (Wagner and Beck 1993) and maize (Takei et al. 2001). These studies suggest that CK metabolism and translocation could be modulated by nitrogen nutritional content and by availability of various nitrogen forms in soil.

In this work, different aspects of ammonium (NH_4^+) toxicity in *Festulolium* and *Trifolium* plants were investigated following their growth with different N forms (ammonium sulphate – $[(NH_4)_2SO_4]$ or ammonium nitrate $[NH_4NO_3]$). The effect of various nitrogen sources on endogenous contents of CKs and their profiles in *Festulolium* and clover grown in pots with moderate NH_4^+ supply were investigated. As far as it is known, this is the first report on rapid responses of plant growth induced by the application of different forms of N by injection application. This study was also focused on reducing the negative effects of ammonium (NH_4^+) toxicity by the presence of NO_3^- in the nutrient solution.

MATERIALS AND METHODS

Experimental setting. The effect of ammonium (NH_4^+) toxicity and beneficial effect of NO_3^- on NH_4^+ nutrition in *Festulolium* and clover were investigated in the present study. For the pot experiments, the seeds of *Festulolium* (cv. Felina PO; Poaceae, *Lolium multiflorum* Lamk. × *Festuca arundinacea* Schreber.) and clover (*Trifolium pratense* L.; cv. Start; Fabaceae) were sown into plastic pots containing soil (10 kg of Chernozem; pH_{KCl} = 7.2, C_{ox} = 1.83%, CEC = 258 $mmol_{(+)}$/kg). After leaves began forming the plants were treated with N (3 g per pot) in the form of ammonium sulphate $[(NH_4)_2SO_4]$ or ammonium nitrate (NH_4NO_3) solution using different type of application (timescale of experiment: (A) 1st year –1. 7. 2008 – sowing of selected species, 2nd year – 4. 5. 2009 – treating of plants by sidedress and injection application; (B) 4. 7. 2009 – sowing and 30. 4. 2010 application of fertilizers). For injection application solution was applied into top soil (100 mm depth) at two points of pot.

The plants 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 (Maximum Water Holding Capacity). The weather was monitored by meteorological station and in accordance with long-term average at this locality. Plants were harvested 1, 3, 5, and 22 days after treatment. Samples were kept frozen in liquid N for transport and then at –80°C until extraction of CKs procedure.

Determination of nitrogen. The dried above-ground biomass and roots were used for determination of total N. 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, Höganäs, Sweden).

Determination of cytokinins. Endogenous CKs were extracted by methanol/formic acid/water (15/1/4, by vol., pH ~ 2.5; –20°C) from samples of *Festulolium* and clover above-ground biomass homogenized in liquid nitrogen and purified using dual-mode solid phase extraction method (Dobrev and Kamínek 2002). CK ribotides were determined as corresponding ribosides following their dephosphorylation by alkaline phosphatase. Detection and quantification were carried out using HPLC/MS (Finnigan, San José, USA) operated in the positive ion full-scan MS/MS mode using a multilevel calibration graph with [2H]-labeled CKs as internal standards. Detection limits of different CKs were between 0.5 and 1.0 pmol/sample.

A wide spectrum of endogenous CKs was revealed by HPLC/MS analysis. For the purpose of this study, they were divided according to their structure and physiological activity into four groups comprising (1) bioactive nucleobases and their ribosides (iP, iPR, Z, ZR, DHZ, DHZR, cisZ, cisZR); (2) CK riboside phosphates (iPRMP, ZRMP, DHZRMP, cisZRMP); (3) storage forms, i.e. *O*-glucosides (ZOG, ZROG, DHZOG, DHZROG, cisZOG, cisZROG) and (4) irreversibly inactive or weakly active N-glucosides (iP7G, iP9G, Z7G, Z9G, DHZ7G, DHZ9G, cisZ7G, cisZ9G).

Statistical analysis. All statistical analyses were performed using hierarchic analyses of variance (ANOVA) with inteactions at a 95% ($P < 0.05$) significance level with a subsequent Tukey's HSD test. Standard deviation was performed from three replicates for every set of data. All analyses were performed by using the Statistica 8.0 software (StatSoft, USA).

RESULTS AND DISCUSSION

Several studies reported that the long-term application of strict NH_4^+ nutrition causes a significant decrease in plant growth, although the intensity of this negative effect depended on the plant species (Belastegui-Macadam et al. 2007). 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). On the other hand, numerous papers showed that the presence of NO_3^- in the nutrient solution corrected the negative effects associated with NH_4^+ nutrition in certain plant species (Houdusse et al. 2007).

Our results showed that $(NH_4)NO_3$ treatment tended to cause a significant increase of the dry matter production in shoots (up to 15%) and roots (up to 20%) compared to NH_4^+-fed plants. The content of total N in above-ground biomass of *Festulolium* exhibited an increasing trend during the 22-day period for both $(NH_4)_2SO_4$ and NH_4NO_3 sidedress as well as injection application. The content of total N in roots followed a similar trend (Table 1).

In *Trifolium pratense* L., the $(NH_4)NO_3$ supply (both sidedress and injection treatment) significantly increased shoot dry matter production. The effect of $(NH_4)NO_3$ on root dry matter was similar to shoots (increase up to 20%). The N content in shoots exceeded that in roots for both $(NH_4)_2SO_4$ and $(NH_4)NO_3$ sidedress as well as injection applications (Table 2). The N content in the above-ground biomass exhibited the same dynamics for both sidedress and injection application peaking 3 days after $(NH_4)_2SO_4$ as well as NH_4NO_3 supply and then sharply decreasing. Similar course in total N content was found in roots after $(NH_4)_2SO_4$ application (both types) while permanently increasing trend within the whole 22-day period was observed following NH_4NO_3 treatments.

It is well known that the accumulation of CKs in plants is in close correlation with their N content as reported for e.g. *Urtica dioica* (Wagner and Beck 1993), barley (Samuelson and Larsson 1993) and maize (Takei et al. 2001). Our results also confirmed this correlation for both species and both sidedress and injection applications (R^2 = 0.66–0.98). Also the impact of N nutrition on the CK levels in plants (Smiciklas and Below 1992, Samuelson and Larsson 1993), on the sterols content (Pavlík et al. 2010a,b) and composition of free amino acids (Neuberg et al. 2010) was well documented.

The CK storage forms, i.e. *O*-glucosides represented major CK derivatives (up to 80% of total CKs) found in *Festulolium* (Tables 3 and 4).

Table 1. The content of N (% DM) in *Festulolium* above-ground biomass and roots during a 22-day period after application of ammonium sulphate and ammonium nitrate

Days after application	Ammonium sulphate		Ammonium nitrate	
	sidedress application	injection application	sidedress application	injection application
Above-ground biomass				
0	1.57 ± 0.17^a	1.63 ± 0.10^a	1.48 ± 0.14^a	1.55 ± 0.18^a
1	1.98 ± 0.15^a	1.75 ± 0.07^a	1.99 ± 0.05^a	2.01 ± 0.09^a
3	2.42 ± 0.08^b	2.20 ± 0.19^a	2.32 ± 0.08^a	2.68 ± 0.11^b
5	2.85 ± 0.09^b	2.93 ± 0.15^b	3.25 ± 0.09^b	3.15 ± 0.09^b
22	3.35 ± 0.10^c	3.20 ± 0.12^b	4.48 ± 0.15^c	3.64 ± 0.08^c
Roots				
0	0.35 ± 0.04^a	0.33 ± 0.04^a	0.40 ± 0.05^a	0.38 ± 0.03^a
1	0.56 ± 0.06^a	0.58 ± 0.03^a	0.72 ± 0.08^b	0.68 ± 0.08^b
3	0.83 ± 0.05^a	0.73 ± 0.08^b	0.71 ± 0.07^b	0.82 ± 0.06^b
5	0.86 ± 0.03^b	0.74 ± 0.11^b	0.94 ± 0.11^b	0.99 ± 0.12^b
22	0.93 ± 0.05^b	0.83 ± 0.09^b	1.25 ± 0.12^c	1.11 ± 0.18^c

Means ± SD of three replications are shown. Data with the same index (in row) represent statistically identical values ($P < 0.05$).

Table 2. The content of N (% DM) in *Trifolium pratense* L. above-ground biomass and roots during a 22-day-period after application of ammonium sulphate and ammonium nitrate

Days after application	Ammonium sulphate		Ammonium nitrate	
	sidedress application	injection application	sidedress application	injection application
Above-ground biomass				
0	3.10 ± 0.17[a]	3.15 ± 0.12[a]	2.96 ± 0.11[a]	3.22 ± 0.10[a]
1	3.45 ± 0.15[a]	4.22 ± 0.19[b]	3.46 ± 0.15[b]	4.04 ± 0.09[c]
3	4.24 ± 0.19[b]	4.24 ± 0.09[b]	4.17 ± 0.14[c]	4.12 ± 0.11[c]
5	3.84 ± 0.21[a]	3.80 ± 0.07[b]	3.84 ± 0.09[b]	3.73 ± 0.21[b]
22	3.04 ± 0.15[a]	2.89 ± 0.06[a]	3.20 ± 0.08[a]	2.82 ± 0.18[a]
Roots				
0	1.75 ± 0.11[a]	1.82 ± 0.23[a]	1.68 ± 0.12[a]	1.85 ± 0.09[a]
1	2.08 ± 0.12[a]	2.17 ± 0.18[b]	2.44 ± 0.08[b]	2.59 ± 0.17[b]
3	2.68 ± 0.09[b]	2.53 ± 0.10[b]	2.44 ± 0.09[b]	2.59 ± 0.16[b]
5	1.86 ± 0.07[a]	2.33 ± 0.09[b]	2.49 ± 0.11[b]	2.60 ± 0.08[b]
22	1.98 ± 0.11[a]	1.33 ± 0.08[a]	3.12 ± 0.21[c]	2.68 ± 0.04[b]

Means ± SD of three replications are shown. Data with the same index (in row) represent statistically identical values ($P < 0.05$)

O-glucosides content is in close correlation with plant N content after sidedress application (the polynomial function of 2nd degree $R^2 = 0.74–0.99$). The levels of other CK derivatives were considerably lower (bioactive nucleobases and their ribosides 4–17%; CK riboside phosphates 1–15% and CK-N-glucosides 5–30% of the total CKs). The dynamics in total CK contents exhibited similar trends for the two NH_4^+ sources and both types of their application. Evidently, the total CK levels reached sharp maxima in *Festulolium* above-ground biomass 5 days after the treatment followed by a strong decrease for all forms of N (Tables 3 and 4).

In contrast to *Festulolium*, the storage of *O*-glucosides did not predominate over other CK forms in *Trifolium plants* (Tables 5 and 6) and their

Table 3. Content of endogenous levels of cytokinins (CK)

Days after application	I	II	III	IV	V
Sidedress application					
1	9.40 ± 0.21[a]	2.54 ± 0.12[a]	123.04 ± 5.32[b]	17.42 ± 0.94[a]	152.40 ± 8.75[a]
3	14.38 ± 0.89[a]	7.46 ± 0.20[a]	86.87 ± 3.33[a]	16.26 ± 0.78[a]	124.97 ± 5.5[a]
5	21.19 ± 1.11[b]	19.23 ± 0.98[b]	220.30 ± 12.5[c]	113.56 ± 4.99[b]	374.28 ± 19.48[b]
22	5.07 ± 0.13[a]	18.27 ± 0.56[b]	89.21 ± 6.21[a]	6.30 ± 3.22[a]	118.85 ± 4.97[a]
Injection application					
1	8.15 ± 0.19[a]	4.02 ± 0.09[a]	122.96 ± 10.19[a]	11.84 ± 1.07[a]	146.97 ± 7.65[a]
3	23.54 ± 1.12[b]	11.20 ± 0.56[a]	119.16 ± 9.77[a]	19.48 ± 1.26[a]	173.38 ± 8.16[a]
5	28.72 ± 0.97[b]	18.70 ± 0.88[b]	158.35 ± 12.13[a]	78.40 ± 8.56[b]	284.17 ± 12.45[b]
22	3.81 ± 0.03[a]	*	61.35 ± 6.45[b]	4.73 ± 0.12[a]	69.89 ± 5.96[c]

I – bioactive nucleobases and their ribosides; II – CK riboside phosphates; III – storage forms. i.e. *O*-glucosides; IV – irreversibly inactive or weakly active N-glucosides; V – total CKs in *Festulolium* above-ground biomass during a 22-day period after application of ammonium sulphate. Values are expressed in pmol/g FW. Means ± SD of three replications are shown. *missing values; data with the same index (in row) represent statistically identical values ($P < 0.05$)

Table 4. Content of endogenous levels of cytokinins (CK)

Days after application	I	II	III	IV	V
Sidedress application					
1	12.85 ± 0.43[a]	4.48 ± 0.09[a]	141.14 ± 12.16[a]	15.71 ± 1.13[a]	174.18 ± 5.62[a]
3	31.41 ± 1.24[b]	19.67 ± 1.45[b]	109.46 ± 10.98[a]	22.08 ± 1.86[b]	182.62 ± 19.86[a]
5	21.77 ± 0.98[a]	10.73 ± 0.87[b]	176.00 ± 18.96[b]	101.99 ± 16.75[c]	310.49 ± 27.86[b]
22	6.87 ± 0.09[c]	*	64.33 ± 6.89[c]	8.83 ± 0.87[a]	80.03 ± 7.99[c]
Injection application					
1	12.29 ± 0.56[a]	5.05 ± 0.74[a]	153.65 ± 14.95[a]	10.23 ± 1.76[a]	181.22 ± 27.97[a]
3	17.43 ± 1.17[a]	7.11 ± 0.86[a]	95.41 ± 7.53[b]	15.49 ± 1.28[a]	135.44 ± 21.98[a]
5	16.98 ± 0.97[a]	2.80 ± 0.05[a]	190.85 ± 20.88[a]	98.57 ± 5.43[b]	309.20 ± 35.93[c]
22	4.87 ± 0.25[b]	*	83.01 ± 4.97[b]	4.67 ± 0.06[c]	92.55 ± 12.75[b]

I – bioactive nucleobases and their ribosides; II – CK riboside phosphates; III – storage forms. i.e. *O*-glucosides; IV – irreversibly inactive or weakly active N-glucosides; V – total CKs in *Festulolium* above-ground biomass during a 22-day period after application of ammonium nitrate. Values are expressed in pmol/g FW. Means ± SD of three replications are shown. *missing values; data with the same index (in row) represent statistically identical values ($P < 0.05$)

content is in close correlation with plant N content after injection application (the polynomial function of 2nd degree $R^2 = 0.89–0.93$) on the contrast of *Festulolium*. The total CK levels in *Trifolium* were higher in case of ammonium nitrate applied sidedress in comparison with other treatments. The more close correlation of CKs content and plant nitrogen content was calculated for injection application. Again, the maximal levels in total CK concentrations were found 5 days after the application. Proportions between individual CK forms remained relatively steady in the course of the experiment (Tables 5 and 6).

To summarize, similar trends in total CK contents with maximum of 5 days after the treatment for both types of application of ammonium sulphate as well as ammonium nitrate were found in *Festulolium* and clover.

Our results are in accordance with Chen et al. (1998) who reported that mixed N nutrition enhances

Table 5. Content of endogenous levels of cytokinins (CK)

Days after application	I	II	III	IV	V
Sidedress application					
1	6.02 ± 0.12[a]	5.79 ± 0.18[a]	6.10 ± 0.14[a]	2.40 ± 0.03[a]	20.31 ± 2.31[a]
3	*	*	*	*	*
5	9.59 ± 0.14[b]	6.62 ± 0.23[b]	11.83 ± 1.11[c]	5.19 ± 0.98[b]	33.23 ± 2.87[c]
22	6.57 ± 0.25[a]	*	8.70 ± 0.52[b]	2.26 ± 0.04[a]	17.53 ± 1.85[b]
Injection application					
1	7.97 ± 0.45[a]	4.36 ± 0.06[a]	11.55 ± 0.87[a]	1.30 ± 0.09[a]	25.18 ± 3.12[a]
3	8.48 ± 0.88[a]	13.43 ± 0.24[b]	10.82 ± 1.12[a]	4.35 ± 0.87[b]	37.08 ± 2.15[b]
5	7.68 ± 0.74[a]	17.04 ± 0.87[c]	11.38 ± 0.74[a]	7.58 ± 0.45[c]	43.68 ± 4.18[c]
22	4.49 ± 0.11[b]	15.92 ± 1.13[b]	10.80 ± 0.24[a]	2.11 ± 0.12[a]	33.32 ± 3.35[b]

I – bioactive nucleobases and their ribosides; II – CK riboside phosphates; III – storage forms. i.e. *O*-glucosides; IV – irreversibly inactive or weakly active N-glucosides; V – total CKs in *Trifolium pratense* L. above-ground biomass during a 22-day period after application of ammonium sulphate. Values are expressed in pmol/g FW. Means ± SD of three replications are shown. *missing values; data with the same index (in row) represent statistically identical values ($P < 0.05$)

Table 6. Content of endogenous levels of cytokinins (CK)

Days after application	I	II	III	IV	V
Sidedress application					
1	8.91 ± 0.53[a]	9.51 ± 0.87[a]	8.70 ± 0.98[a]	1.41 ± 0.58[a]	28.53 ± 2.15[a]
3	14.98 ± 1.12[b]	14.04 ± 1.11[b]	20.09 ± 2.12[b]	3.24 ± 0.45[b]	52.35 ± 4.87[b]
5	18.28 ± 1.18[c]	12.67 ± 1.10[b]	7.32 ± 1.10[a]	15.04 ± 1.12[c]	53.31 ± 5.02[b]
22	5.68 ± 0.87[d]	*	9.25 ± 0.87[a]	1.50 ± 0.09[a]	16.43 ± 1.12[c]
Injection application					
1	7.87 ± 0.21[a]	5.30 ± 0.07[a]	8.81 ± 0.87[a]	2.69 ± 1.10[a]	24.67 ± 2.23[a]
3	4.76 ± 0.15[b]	9.12 ± 0.45[b]	1.86 ± 0.05[b]	1.66 ± 0.98[a]	17.40 ± 1.15[b]
5	6.95 ± 0.35[a]	20.53 ± 1.15[c]	16.75 ± 1.08[c]	5.74 ± 0.89[b]	49.97 ± 5.48[c]
22	4.85 ± 0.87[b]	*	9.49 ± 0.87[a]	2.26 ± 0.45[a]	16.60 ± 2.01[b]

I – bioactive nucleobases and their ribosides; II – CK riboside phosphates; III – storage forms. i.e. *O*-glucosides; IV – irreversibly inactive or weakly active N-glucosides; V – total CKs in *Trifolium pratense* L. aboveground biomass during a 22-day period after application of ammonium nitrate. Values are expressed in pmol/g FW. Means ± SD of three replications are shown. *missing values; data with the same index (in row) represent statistically identical values ($P < 0.05$)

growth in wheat plants. In our study, the application of NO_3^- to NH_4^+-fed plants was also associated with a recovery in absolute growth rate (AGR), both in roots and shoots. This beneficial effect observed in amonium nitrate-fed plants was also well correlated with an increase in plant N content which was significantly enhanced in shoots. Interestingly, the beneficial effect of NO_3^- treatment in improving plant dry weight and N content was dependent on the type of application of N supplied.

In summary, our results indicate that the negative effect of application of NH_4^+ on the growth of *Festulolium* and clover plants fed could be modulated by NO_3^-. The presence of NO_3^- was associated with changes in the forms of CKs, dependently on the type of N-application. Likewise, the presence of NO_3^- also enhanced N shoot content which correlated with higher CK levels. The same effects were observed at either sidedress or injection application dose of N thus supporting the possible role of NO_3^- as a signal molecule involved in promoting plant growth and its beneficial effects that can reduce NH_4^+ toxicity in *Festulolium* and clover.

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