

Interactions between nitrogen nutrition and phytohormone levels in *Festulolium* plants

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ABSTRACT

This study aims to reveal and to compare effect of two various systems of nitrogen nutrition, namely injection and sidedress application, on the levels of NO_3^- and phytohormones (cytokinins, abscisic acid (ABA), indole-3-acetic acid (IAA)) in *Festulolium* plants. Calcium nitrate (CN) or ammonium sulphate (AS) were used as N source in the pot experiment. The yield of *Festulolium* above and below ground biomass was more substantially enhanced after sidedress application of both AS or CN in comparison with injection application. The levels of endogenous cytokinins peaked 5 days after application regardless the form of N source and the method of application. The increased concentration of endogenous cytokinins as well as the enhanced content of selected free amino acid suggest that N applied sidedress can maintain better distribution patterns of nutrients and provide improved conditions for growth and nitrogen uptake. The nitrogen application affected ABA concentrations on all tested treatments. The correlation between NO_3^- and ABA concentrations ranged from 0.77 to 0.89. A significant IAA increase was recorded in plants after ammonium nitrogen sidedress application and it was confirmed that applications of nitrate reduced IAA concentration (correlation between NO_3^- and IAA for AS treatment $R^2 = 0.77-0.94$).

Keywords: abscisic acid; amino acid; CULTAN; cytokinin; indole-3-acetic acid; Poaceae

Nitrogen is a macronutrient present in many key biological molecules. It is available for plants predominantly as NO_3^- and NH_4^+ . It controls many aspects of plant life, and has a strong impact on plant development. In response to changes in nitrogen supply, plants display elaborate responses at both physiological and morphological levels to adjust their growth and development (Vidal and Gutierrez 2008, Lošák et al. 2010). The reduction of NO_3^- to ammonium (NH_4^+) involves the sequential action of nitrate reductase and nitrite reductase. The resulting NH_4^+ is then assimilated by glutamine synthetase and glutamate synthase to organic forms such as glutamate (Glu) and glutamine (Gln). These amino acids (AAs) are precursors in the synthesis of other AAs, nucleic acids, chlorophylls or hormones.

The question of how N supply modifies the hormonal status of plants was studied by many scientists

(reviewed in Krouk et al. 2011). Amongst phytohormones, abscisic acid (ABA), indole-3-acetic acid (IAA), and cytokinins (CK) were closely linked to nitrogen signaling (Kiba et al. 2011). Many studies highlighted the close correlation between nitrogen and CK in several plant species (Takei et al. 2001, 2002, Cline et al. 2006). The interactions between N and CK in the regulation of metabolism and development revealed that CK biosynthesis is up-regulated by nitrate and the hormone in turn was proposed as a regulator of the morphological response, protein synthesis capacity and macronutrient acquisition (Sakakibara et al. 2010). These findings unequivocally indicate modulation of CK metabolism and translocation by the N nutritional status.

The results of Garnica et al. (2010) showed that the presence of NO_3^- was associated with clear increases in the active forms of CK (zeatine, trans-zeatine riboside, isopentenyl adenosine)

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and reduction of the levels of the lower active forms (cis-zeatine riboside), independently of the N dose applied. The presence of nitrate also enhanced IAA shoot content, which correlated with higher CK levels and a tendency toward lower ABA concentration.

The present study is focused on N nutrition and hormone relationships, mainly on a question whether phytohormone levels of the *Festulolium* can be changed not only by an addition of different N forms (ammonium or nitrate nitrogen), but also different application procedure (sidedress or injection application).

MATERIAL AND METHODS

Festulolium (cv. FELINA^{PO}; Poaceae, *Lolium multiflorum* Lamk. × *Festuca arundinacea* Schreber.) seeds were germinated and plants were cultivated in pots (20 plants per pot containing 10 kg of Chernozem – Suchdol; Table 1) in the outside vegetation hall (Czech University of Life Sciences Prague, Czech Republic) under natural light and temperature conditions. 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 (AS; 21% N, 24% S) or calcium nitrate (CN; 15% N, 20% Ca) treatments using either (1) sidedress application or (2) injecting N application (Neuberg et al. 2011). Nitrogen fertilizer was applied to the developing plants after leaves began to form, namely at rates 3 g of N per pot by sidedress application or into top soil, 100 mm depth, on two points of pot by injecting application). Each treatment was performed in five replications. Plants (leaves and roots) were harvested 1, 3, 5, 22, 60 and 125 days after treatment. Samples were

frozen in liquid nitrogen for transport and stored at -80°C until next extraction procedure.

The dried aboveground biomass was used for determination of total N and nitrate 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, Höganäs, Sweden). For nitrate analyses dried aboveground plant biomass and roots were extracted by distilled water (1:10, w/v). Contents of N-NO_3^- levels were determined by segmental flow-analysis using a colorimetric method on a SKALAR^{plus} SYSTEM (Skalar, Breda, Netherlands).

Total amino acid compounds were determined using an EZ-faast amino acid analysis procedure (Phenomenex, Torrance, USA). Samples were analyzed for amino acid contents by the gas chromatography coupled with mass spectrometry detection using a HP 6890N/5975 instrument (Agilent Technologies, Santa Clara, USA; Pavlík et al. 2010a).

Phytohormone concentrations (ABA, IAA, CK) were analysed following extraction in Bielecki's modified solvent (Hoyerová et al. 2006) and purification by cation exchange and reverse-phase chromatography using Si-C₁₈ and Oasis MCX mixed mode columns (Dobrev and Kamínek 2002). Identification and quantification of plant hormones were achieved using two-dimensional liquid chromatograph (Ultimate 3000, Dionex, Germering, Germany) coupled to hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, Carlsbad, USA).

RESULTS AND DISCUSSION

Various fertilizing systems and their relationship to N (NH_4^+ or NO_3^-) transport were assessed

Table 1. Nitrogen contents in above ground dry biomass (DW) of *Festulolium* ($n = 10$, i.e. two experimental years and five replication per each year)

Days after application	Content of total N (%)			
	ammonium sulphate (AS)		calcium nitrate (CN)	
	sidedress application	injection	sidedress application	injection
1	2.13 ± 0.23	1.98 ± 0.49	2.99 ± 0.35	1.55 ± 0.26
3	2.41 ± 0.27	2.31 ± 0.41	3.00 ± 0.62	2.74 ± 0.21
5	2.85 ± 0.52	3.24 ± 0.49	3.36 ± 0.38	3.24 ± 0.22
22	4.35 ± 0.74	4.47 ± 0.69	4.23 ± 0.35	3.81 ± 0.51
60	3.85 ± 0.66	3.63 ± 0.19	3.78 ± 0.21	3.65 ± 0.69
125	3.52 ± 0.67	3.52 ± 0.15	3.45 ± 0.45	3.47 ± 0.71

Table 2. Nitrate content in above ground and root dry biomass (DW) of *Festulolium* ($n = 10$, i.e. two experimental years and five replication per each year)

Days after application	Content of NO_3^- (mg/kg DW)			
	ammonium sulphate (AS)		calcium nitrate (CN)	
	sidedress application	injection	sidedress application	injection
Above ground biomass				
1	176 ± 27	180 ± 36	8141 ± 218	236 ± 69
3	4568 ± 398	1123 ± 197	15328 ± 457	1836 ± 361
5	16476 ± 418	2349 ± 293	40353 ± 589	4219 ± 215
22	24654 ± 278	4047 ± 254	85456 ± 648	20145 ± 411
60	30456 ± 349	4622 ± 116	70256 ± 742	40465 ± 546
125	50446 ± 166	5187 ± 238	65234 ± 439	30465 ± 615
Roots				
1	905 ± 79	1008 ± 56	583 ± 91	727 ± 62
3	1719 ± 115	1160 ± 531	2968 ± 316	1108 ± 101
5	2284 ± 284	2921 ± 195	5468 ± 203	5426 ± 254
22	9875 ± 367	5643 ± 164	7857 ± 207	10237 ± 423
60	10665 ± 341	10467 ± 297	8242 ± 154	11235 ± 407
125	20466 ± 328	7455 ± 211	6542 ± 176	9642 ± 237

in the present study to follow the dynamics of N uptake; namely (1) a local injection application and (2) a sidedress application.

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 sidedress application of both AS or CN treatments (167.5 g FW and 171.4 g FW per pot, respectively) in comparison with injection application (140.3 g FW and 141.4 g FW, respectively). The significant difference between AS and CN treatments for roots biomass was not confirmed (data not shown). According to Celis-Aramburo et al. (2011) inhibition of primary root growth was observed by exposing the root to nitrate nitrogen; in contrast, ammonium was not able to induce similar changes.

Our previous results confirmed yield decrease after nitrogen injection application (Neuberg et al. 2010, Pavlík et al. 2010a). This contradicts the results of Kozlovský et al. (2010) in field experiments, who obtained higher yields in treatments with injection fertilization of grass-clover mixture.

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, were found. For all four treatments, the contents

of nitrogen increased markedly between the start of vegetation and flowering and then tended to decrease slightly or to remain constant up to maturity (Table 1). Our observations are in agreement with the results of Kozlovský et al. (2010).

From the present results, concentration of nitrate ions as a function of added nitrogen and type of application varied considerably (Table 2). Despite a similar progressive increase in the NO_3^- concentration in plant biomass, the sidedress application resulted in significantly higher NO_3^- levels compared to injection application throughout the 125 days after application. It was concluded that the injection method became inferior opposite to sidedress application which maintained an optimal growth conditions for longer time.

Nitrogen flow through the amino acids can change dramatically in response to N fertilization. Glutamic acid (Glu), aspartic acid (Asp) and asparagine (Asn) are used to transfer nitrogen from source organs to sink tissues and to build up reserves during periods of nitrogen availability for subsequent use in growth, defense, and reproductive processes. The changes of free amino acid concentrations after N application are demonstrated in Figure 1. The significant increase of Glu content was observed after injection application of CN and contents of Asp and Asn were affected

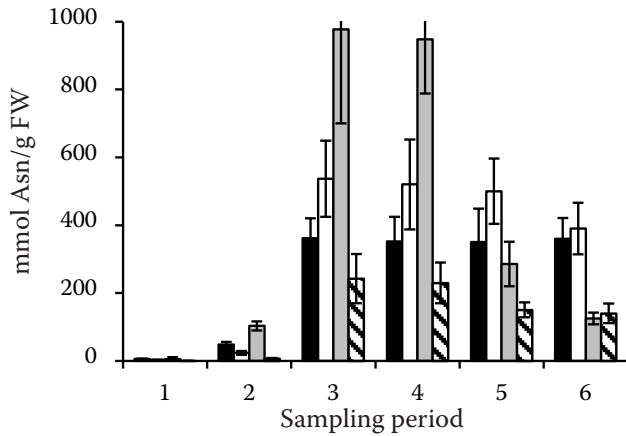
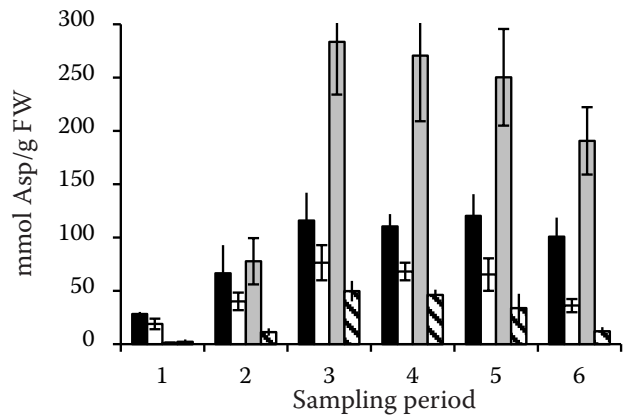
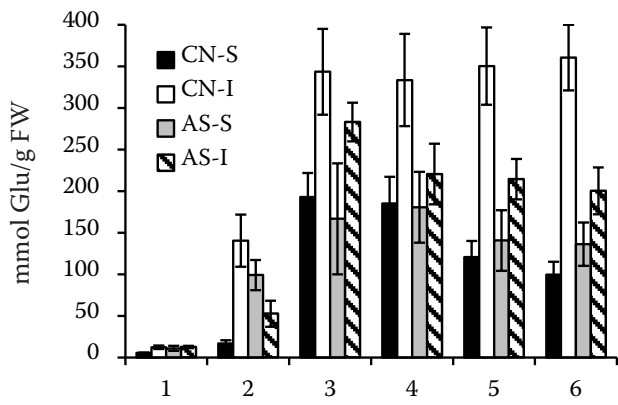


Figure 1. The contents of transfer of amino acids glutamic acid (Glu), aspartic acid (Asp) and asparagine (Asn) in above ground biomass of *Festulolium* (Sampling period 1 = 1 day; 2 = 3 days; 3 = 5 days; 4 = 22 days; 5 = 60 days and 6 = 125 days after treatment; $n = 10$, i.e. two experimental years and five replication per each year)

mainly by ammonium nitrogen (sidedress application of AS). The concentrations of these amino acids showed linear correlations with NO_3^- concentration in aboveground biomass – $R^2 = 0.82-0.93$ for both AS treatments, $R^2 = 0.48-0.65$ for both CN treatments. The amino acids proline (Pro) and β -alanine (Ala) accumulates markedly in response to stress in plants (Pavlík et al. 2010b). The increase of free Pro content in aboveground biomass was detected in our experiment, mainly in roots and aboveground biomass of AS treatments (Figure 2),

and for these treatments correlations between Pro and NO_3^- concentration in roots and aboveground biomass was calculated $R^2 = 0.94-0.99$. Strong correlation between NO_3^- concentration and Ala was mainly determined in roots ($R^2 = 0.74-0.97$) and the strongest correlation was observed on AS treatment after injection application. The injection application of AS showed the most significant effect on contents of selected free amino acids in roots and aboveground biomass in contrast to the other treatments. According to Atanasova (2008)

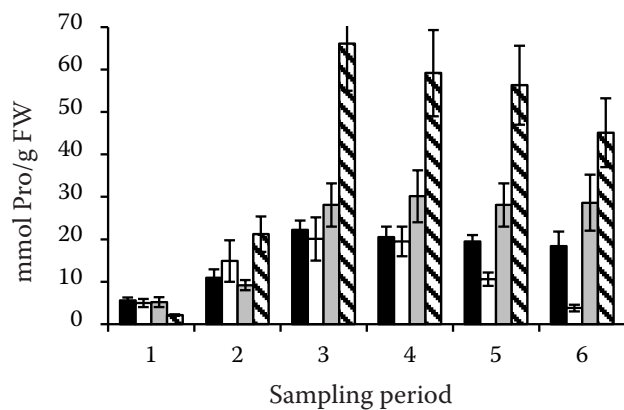
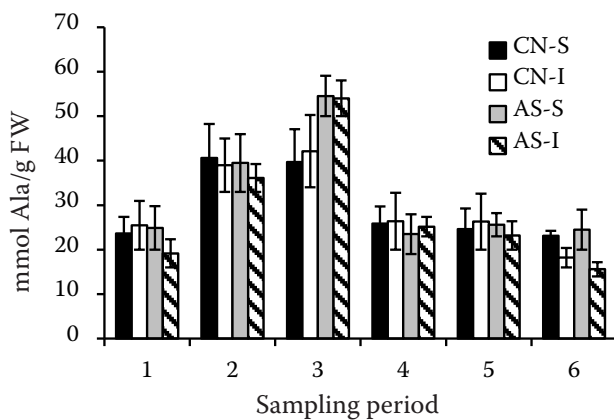


Figure 2. The contents of alanine (Ala) and proline (Pro) in above ground biomass of *Festulolium* (Sampling period 1 = 1 day; 2 = 3 days; 3 = 5 days; 4 = 22 days; 5 = 60 days and 6 = 125 days after treatment; $n = 10$, i.e. two experimental years and five replication per each year)

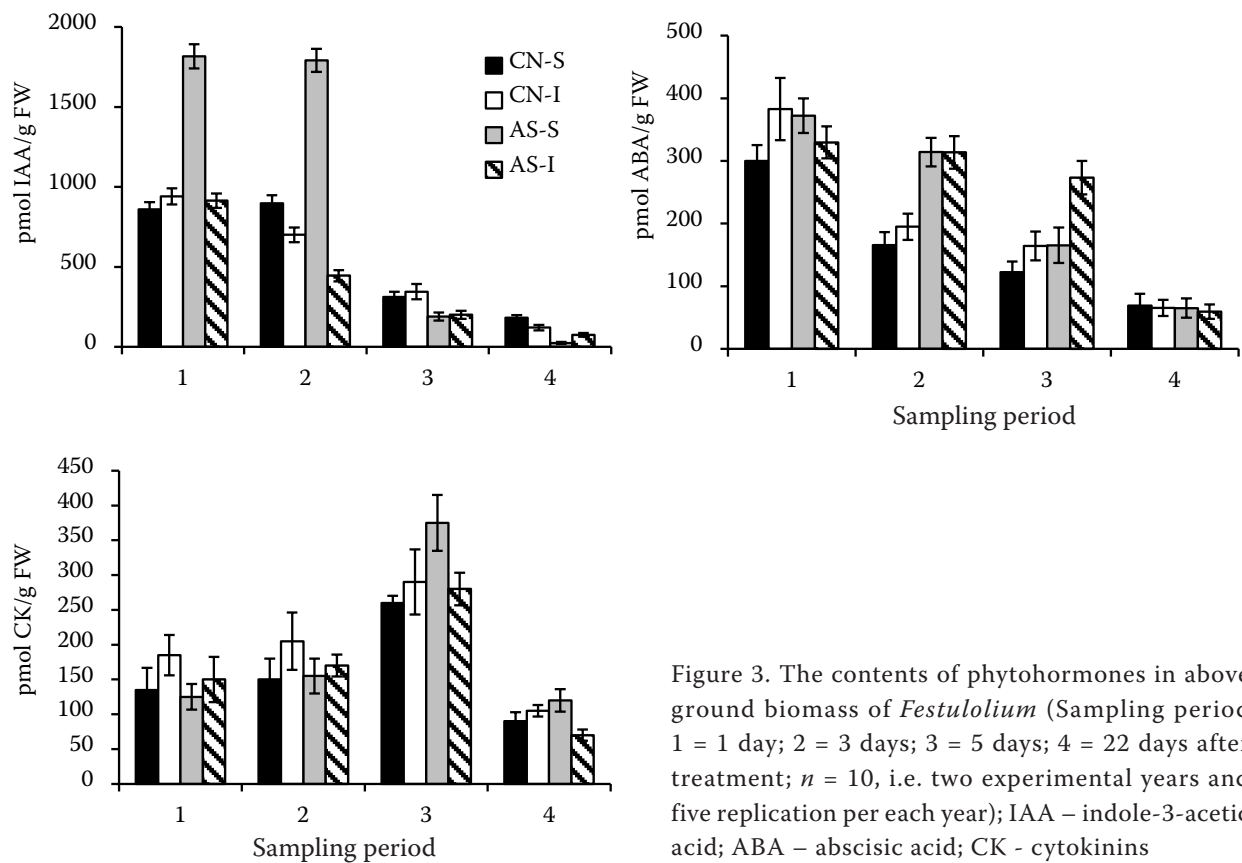


Figure 3. The contents of phytohormones in above ground biomass of *Festulolium* (Sampling period 1 = 1 day; 2 = 3 days; 3 = 5 days; 4 = 22 days after treatment; $n = 10$, i.e. two experimental years and five replication per each year); IAA – indole-3-acetic acid; ABA – abscisic acid; CK - cytokinins

the increase of proline and alanine could serve as an indicator for unbalanced nitrogen nutrition.

The nitrogen application affected similarly ABA concentrations on all tested treatments (Figure 3). The correlation between NO_3^- and ABA concentrations ranged from 0.77 to 0.89. The lower reductions of ABA concentrations 3 and 5 days after fertilization were observed after ammonium nitrogen application, mainly after injection application. Abscisic acid is thought to play an important role in proline accumulation (Ku et al. 2012). Our results are in agreement with this observation. The correlations between ABA and Pro contents of AS treatments are significant ($R^2 = 0.89-0.98$).

Significant IAA increase was recorded in plants after ammonium nitrogen sidedress application (Figure 3). IAA shoot content of this treatment correlated with nitrate content in roots and above ground biomass ($R^2 = 0.77$ and 0.94). According to Liu et al. (2010) applications of nitrate reduced shoot-to-root IAA transport and decreased IAA concentration in roots to a level more suitable for lateral-root growth.

The total CK levels exhibited a sharp maximum in *Festulolium* aboveground biomass 5 days after application of all forms of N. Then, a strong decrease (to the initial values prior to N application occurred (Figure 3). The contents of CKs in aboveground biomass closely correlated with

NO_3^- contents in roots and aboveground biomass of all treatments ($R^2 = 0.57-0.76$). According to Argueso et al. (2009) NO_3^- applications resulted in increased shoot CKs and the activation of genes associated with nutrient uptake and homeostasis. Glu and Asp are key amino acids for biosyntheses of cytokinins and correlation of their concentrations and CK were close (for CN $R^2 = 0.53-0.7$ and AS treatments $0.97-0.99$).

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