# Infrared spectroscopy-based metabolomic analysis of maize growing under different nitrogen nutrition

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**ABSTRACT**

For metabolomic analysis of maize plants growing under different nitrogen nutrition sequential extraction of fresh biomass was used and isolated fractions were characterized and evaluated using IR spectra. The IR spectra of individual fractions were evaluated in relation to the nitrogen rates (2 g or 4 g N), to applied fertilizers (ammonium nitrogen or urea ammonium nitrate solution) and sampling period. For butanol fraction, typical bands of flavonoids, polar phospholipids, steryl glycosides, analogues of ecdysteroids were characterized. The IR spectra of BuOH fraction showed changes of relative contents of isolated compounds mainly affected by nitrogen rates. For water fraction bands of organic acids, salts of organic acids, flavonoid glycosides and oligopeptides (phytochelatins and/or glutathione) were the most significant. The results showed an increased induction of oxalic acid in plants after 4 g N application. Degradation of this acid induced oxidative stress, therefore strong correlation among contents of oxalic acid, flavonoids and compounds with amide nitrogen (glutathione) was observed in plants growing under 4 g nitrogen nutrition. The glutathione-ascorbate cycle protects plants against oxidative damage.

**Keywords**: ammonium; ecdysone phosphate; CULTAN; sequential extraction; *Zea mays*

Ammonium ($NH_4^+$) is one of the major nutrients for plants, and a ubiquitous intermediate in plant metabolism. However, this ion is notorious for its stress effects on plant species (Kronzucker et al. 2001). Besides growth reduction, several charach teristics of plant metabolism – lower content of mineral cations and organic anions and higher levels of amino acids – are altered, resulting in the so-called 'ammonium syndrome'.

Ammonium dissipated transmembrane proton gradient that is required for both photosynthesis and respiratory electron transport and for sequestering metabolites in the vacuole. Cytoplasmatic ammonium toxicity might lead to rapid necrosis of the plant tissue. Besides controlling ammonium uptake across the plasma membrane, plant cells can modulate ammonium efflux out of the cytoplasm, ammonium compartmentation into the vacuole, or ammonium assimilation in the cytoplasm or the plastids (Loqué and von Wirén 2004). The stress effect initiates plant defensive mechanisms with subsequent metabolic changes. Amino acid metabolism has the central role in stress resistance of plants. The formation of amino acids, amides and related compounds is the main pathway of detoxification of ammonium ions (Britto and Kronzucker 2002). Plants evolve various protective mechanisms to eliminate or reduce reactive oxygen species, which includes enzymes such as ascorbate peroxidase, superoxide dismutase, glutathione reductase, catalase. Plant phenolic compounds may also act as antioxidants or prooxidants in different reactions (Kähkönen et al. 1999, Domingues-Valdivia et al. 2008).

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Recent progress in plant biology has provided the means to tackle complex plant nutritional problems through genomic, transcriptomic, proteomic and metabolomic approaches. All these approaches enable to elucidate the functions and interactions of plant nutrients at the molecular, cellular, organ and whole-plant levels (Yan et al. 2006, Cevallos-Cevallos et al. 2009). Plant nutriomics is a new frontier of study that integrates nutritional functions at various levels with genomics, transcriptomics, proteomics and metabolomics tools of analysis. Metabolomic analyses consist of a sequence of steps including sample preparation, metabolite extraction, derivatization, metabolite separation, detection, and data treatment. Plant metabolomics uses various analytical methods, for example infrared spectroscopy (IR; Pavlíková et al. 2002). Transcriptomic and metabolomic approaches were used to study the impact of nitrogen and sulphur deficiency on N and S remobilization from senescing canopy tissues (Howarth et al. 2008), plant metabolite profiling under nutrient deprivation (Bölling and Fiehn 2005), potato tuber life cycle (Shepherd et al. 2010).

The aim of this paper is the use of IR spectra for characterization of types of compounds in isolated fractions, but also evaluation of proportions of these compounds in relation to metabolism changes after application of nitrogen fertilizers. The water and butanol fractions isolated by sequential extraction of fresh maize biomass are studied in detail, because the changes of compounds in water fraction are affected by nitrogen fertilizers (amino acids and acids of citrate cycle), while phenolic compounds and no active ecdysteroid phosphates are contained in butanol fraction.

## MATERIAL AND METHODS

**Plant material and cultivations**. The effect of nitrogen nutrition for maize metabolism (*Zea mays* L. of hybrid Rivaldo) was investigated in pot experiment. Chernozem soil mixture ($pH_{KCl}$ = 7.2, $C_{ox}$ = 1.83%, CEC = 258 $mmol_{(+)}$/kg) was thoroughly mixed with N dose (2 or 4 g N per pot) applied in the form of ammonium nitrate (AN) for control treatments or was left without nitrogen for treatments with local nitrogen application. Nitrogen in the liquid form of urea ammonium nitrate (UAN) solution was applied into top soil (100 mm depth) at two points of pot 30 days after maize sowing (CULTAN method; Kozlovský et al. 2009). Each treatment was performed in five replications (Table 1). The aboveground biomass of maize was sampled after 3 and 13 days after nitrogen application. This pot experiment was described in detail by Pavlík et al. (2010a).

**Sequential extraction of maize biomass**. Sequential extraction of fresh maize biomass was conducted according to the extraction scheme shown in Figure 1. Four fractions were isolated – petrol ether, butanol, water fractions and non-extractable residues. Isolated fractions – butanol (BuOH) and water extracts were characterized using IR analyses.

Ultra-pure redistilled MeOH (Fluka), redistilled water and all chemicals (Fluka) of analytical grade (p.a.) were used for sample analyses and isolations.

**Analysis of infrared spectra and proportion evaluation of compounds contained in water and butanol fractions**. Isolated fractions were characterized using IR spectra, which were measured transmittance (%) using an IR spectrometer (Bruker IFS 88). The individual isolated fractions were pressed into KBr pellets (∅ 10 mm; sample weight about 1 mg) under 6.86 MPa pressure, with evacuation by oil rotary vacuum pump. Method of IR analysis was described in detail by Pavlíková et al. (2005).

Typical bands of functional groups of organic compounds (flavonoids, polar phospholipids, steryl glycosides, analogues of ecdysteroids, organic acids, and salts of organic acids, oligopeptides (phytochelatins, glutathione) and flavonoid glycosides) were determined. For quantitative analysis the intensities of individual bands ($cm^{-1}$) were calculated and they were divided by intensity of band finding in all treatments. These quotients were used for the relative weight comparison of analyzed substances.

Table 1. Design of the experiment

| Treatment | N rate (g per pot) | Application time |
|---|---|---|
| Ammonium nitrate 1 (AN1) | 2 | before sowing |
| Urea ammonium nitrate solution 1 (UAN1) | 2 | 30 days after maize sowing |
| Ammonium nitrate 2 (AN2) | 4 | before sowing |
| Urea ammonium nitrate solution 2 (UAN2) | 4 | 30 days after maize sowing |

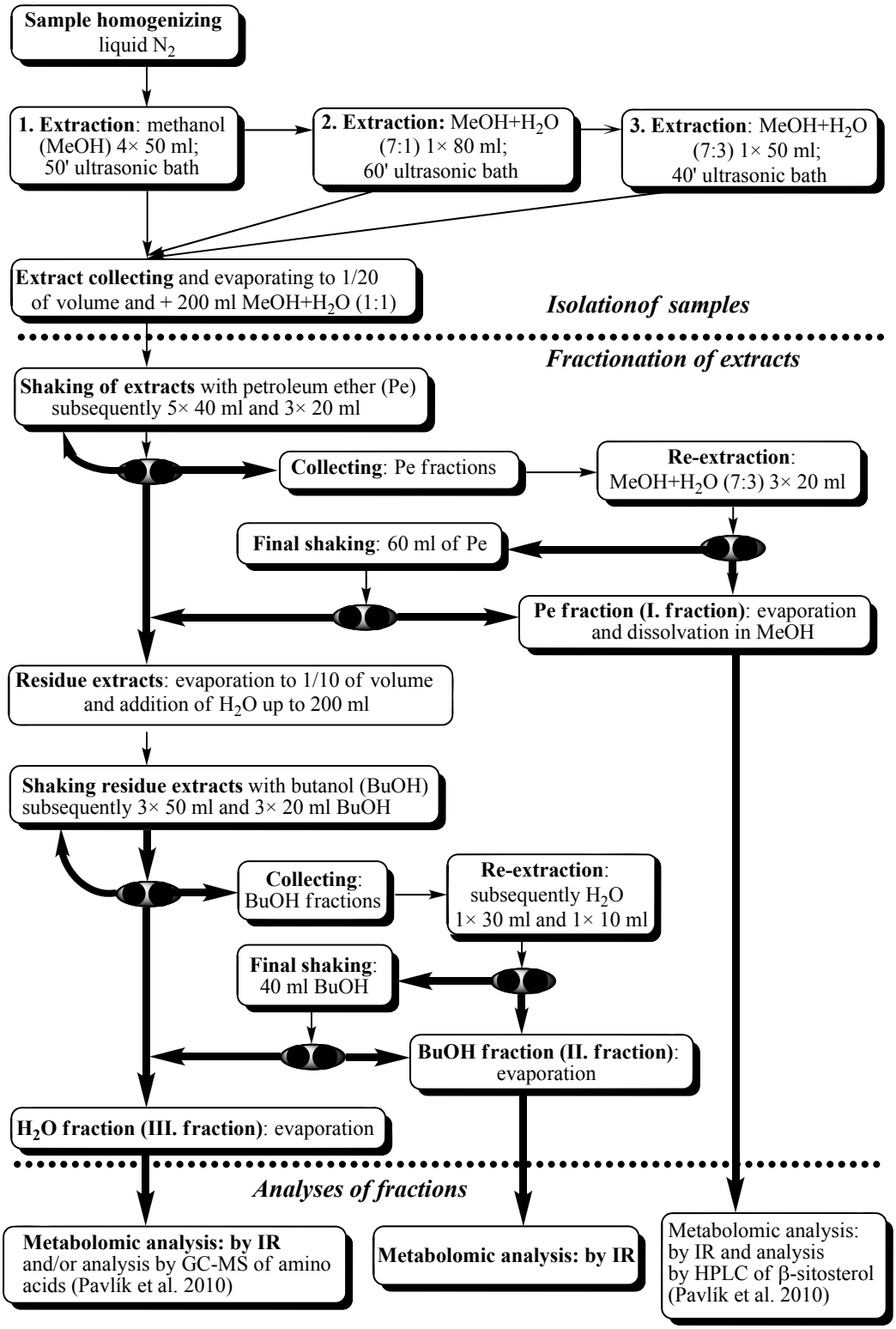


Figure 1. Sequential extraction scheme of maize aboveground biomass

The band $\nu_{C=O}$ 1733/cm (ester bond) was used for detection of relationship between compounds in butanol fraction [conjugated ketone – typical for analogues of ecdysteroids (CK-AE) and conjugated ketone–flavonoids (CK-F), aromatic substances–flavonoids (AS-F), glycosyl substancs es–steryl glycosides (GS-SG) or CK-F and GS-SG]. Conjugated ketone bond ($\nu_{C=O}$ 1623/cm) in this fraction was used for detection of relationship between ester substances–polar phospholipids (ES-PPhL) and CK-AE, AS-F, GS-SG, CK-AE. For detection of relation of compounds in water

Table 2. The portion of isolated fractions from experimental treatments (%) and from the 1st and 2nd sampling periods

| Fraction | AN1 | | UAN1 | | AN2 | | UAN2 | |
|---|---|---|---|---|---|---|---|---|
| Sampling period | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Petroleum ether | 0.6 | 1.5 | 3.8 | 2.0 | 2.7 | 1.7 | 2.3 | 0.8 |
| BuOH | 4.8 | 4.1 | 4.7 | 3.4 | 3.5 | 4.3 | 4.2 | 5.9 |
| $H_2O$ | 10.2 | 11.2 | 9.4 | 10.9 | 11.0 | 11.1 | 10.1 | 10.7 |
| Non-extractable residues | 84.3 | 83.2 | 82.1 | 83.7 | 82.7 | 82.9 | 83.4 | 82.6 |
| Σ nonpolar fractions | 5.5 | 5.6 | 8.5 | 5.4 | 6.2 | 6.0 | 6.5 | 6.7 |
| Σ extractable compounds | 15.7 | 16.8 | 17.9 | 16.3 | 17.3 | 17.1 | 16.6 | 17.4 |

fraction [organic acids (OA) and amides, aromatic substances–flavonoids glycosides (AS-FG), salts of organic acids (SOA), or amides and conjugated ketone–flavonoids glycosides (CK-FG), AS-FG, SOA and/or CK-FG and SOA and/or AS-FG and SOA] the band $\nu_{C\text{-}O}$ 1052/cm (ester bond) was used.

For calculation of changes of substance different groups of linear and polynomial functions of the 2nd degree were used.

## RESULTS AND DISCUSSION

The content of isolated extracts from plant biomass was affected by nitrogen nutrition (Table 2). From Table 2 we can see that weights of isolated fractions – non-extractable residues and Σ extractable compounds – have low variation coefficient ($V_x$ = 0.8; 4.2%). The low variation was also calculated for total extracted weights of experimental treatments. The highest variation coefficient ($V_x$ = 53.6%) was determined for petroleum ether (Pe) extract. The highest variation coefficient is given by sensitivity of plants against oxidative stress induced by $NH_4^+$ (Morita et al. 2004). Stress affected the contents of isolated substances – their decrease in Pe fraction and their increase in water fraction. The increased levels of amino acids contained in water fractions confirmed Pavlík et al. (2010a) and Neuberg et al. (2010). Pavlík et al. (2010a) also published differences of content of β-sitosterol and amino acids under ammonium nitrogen stress. Acetyl-CoA is starting material for biosynthesis of fatty acids, several amino acids, acids of Krebs' cycle (citrate cycle), sterols including their glycosides and phytoecdysteroids and provides a link between the substances contained in BuOH and water fractions. Lange and Ghassemian (2003) focused research on the isoprenoid biosynthetic pathway providing intermediates for the synthesis of a multitude of natural products which serve numerous biochemical functions in plants, for example sterols, and discussed the importance of acetyl-CoA in the different pathways. Their results confirmed our findings. The high correlation between isolated fractions showed their close relationship (Table 3).

The contents of individual compounds of tested treatments differed, but reciprocal regulation of this compound metabolism existed in relation to nitrogen nutrition. This regulation can be characterized by three relations of compounds: (1) the contents of both groups of compounds are increased (direct proportion); (2) the contents of both groups of compounds are decreased (inverse proportion); (3) the contents of both groups of compounds are not significantly changed. Metabolomic analysis is a suitable method for evaluation of relationships between hundreds of compounds contained in isolated extracts (Bölling and Fiehn 2005).

Analyses of IR spectra were used for characterization of typical bands of functional groups of organic compounds in isolated extracts (Table 4). For butanol fraction typical bands of flavonoids, polar phospholipids, steryl glycosides, analogues of ecdysteroids were characterized. For water fraction bands of organic acids, salts of organic acids, flavonoid glycosides and oligopeptides (phytochelatins and/or glutathione) were the most significant.

Coefficients of determination (Tables 5 and 6) confirmed the significant nitrogen effect on biosynthesis regulation of evaluated substances. The IR spectra of individual fractions were evaluated in relation to the nitrogen rates (2 g or 4 g N), to applied fertilizers (ammonium nitrogen or urea ammonium nitrate solution) and sampling period.

The IR spectra of BuOH fraction showed changes of relative contents of isolated compounds mainly affected by nitrogen rates and nitrogen fertilizers (Table 5). The results did not confirm a significant effect of sampling periods. Very closed linear

Table 3. The relationship between isolated fractions of individual treatments characterised by coefficient of determination $R^2$ using polynomial function of the 3rd degree ($R_3^2$)

| Effect of | $R_3^2$ | Effect of | $R_3^2$ |
|---|---|---|---|
| Pe extract on the BuOH extract | 0.678 | BuOH extract on the Pe extract | No*1 |
| Pe extract on the $H_2O$ extract | 0.686 | $H_2O$ extract on Pe extract | 0.629 |
| Pe extract on the ERB*2 | 0.517 | ERB*2 on the Pe extract | 0.721 |
| $H_2O$ extract on the ERB*2 | 0.592 | ERB*2 on the $H_2O$ extract | 0.725 |
| NPE on the $H_2O$ extract | 0.661 | $H_2O$ extract on the NPE | 0.803 |
| NPE on the ERB*2 | 0.730 | ERB*2 on the NPE | 0.894 |

ERB – extraction residue of biomass isolated after extraction; NPE – sum of non-polar extracts (petroleum ether extract and BuOH extract); *1 coefficients of determination ($R_3^2$) characterising significant dependence are only published here; *2 coefficients of determination ($R_3^2$) are the identical for sum of extracts

correlations between contents of ecdysteroids and flavonoids ($R^2$ 0.96–0.99) or polar phosphor lipids and steryl glycosides ($R^2$ 0.89–0.91), or polar phospholipids and analogues of ecdysteroids ($R^2$ 0.68–0.69) were observed in relation to nitroi gen rate. A decrease of linear relation of isolated compounds was mostly observed after 4 g N rate in contrast to 2 g N. The decrease of linear dependence of flavonoids and steryl glycosides was from $R^2$ 0.72 (2 g N rate) to 0.55 (4 g N rate) or polar phospholipids and analogues of ecdysteroids from $R^2$ 0.94 to 0.82. The polynomial function of the 2nd degree showed strong dependence for both nitrogen rates, especially for 4 g N rate (Table 5).

Table 4. The main groups of compounds in BuOH and $H_2O$ fractions characterized by IR spectra (BuOH – butanol fraction)

| IR bands ($cm^{-1}$) | The main groups IR bands ($cm^{-1}$) of compounds in BuOH fraction |
|---|---|
| $\nu_{AROM.}$ 1513–1515 | **flavonoids** (secondary metabolism) and interconnected bands: $\nu_{C=O}$1622–1623 conjugated ketone (**flavonoids**), $\nu_{OH}$ 3396–3417, $\nu_{C-O}$ 1268–1273, $\delta_{CH\text{-}arom.}$ 785–786, bands $\nu_{AROM.}$ 1513–1515 can be connected with aromatic ring of tocopherols |
| $\nu_{C=O}$ 1726–1733 | ester binding – **polar phospholipids** and interconnected bands: $\nu_{C-O}$ 1164–1165 ester, $\nu_{PO}$ 984–985(sh 985); $\nu_{P=O}$ 1268–1273, $\nu_{CH}$ 2926–2928; $\nu_{CH}$ 2853–2855 and $\delta_{(CH2)n}$ $n \geq 4$ 719–724 |
| $\nu_{C-O}$ 1070–1071 | **steryl glycosides** and interconnected bands: $\nu_{C-O}$ 1033 – sh 1035 (saccharide – secondary alcohol), $\nu_{CH}$ 2926–2928; $\nu_{CH}$ 2853–2855, $\nu_{OH}$ 3396–3417 |
| $\nu_{C=O}$ 1652 | conjugated ketone – typical for **analogues of ecdysteroids**; in maize for example ecdysteroid phosphates – 20-hydroxyecdysone phosphate and interconnected bands $\nu_{C-O}$ and/or phosphate 1033 – sh 1035, $\nu_{PO}$ 984–985 (sh 985), $\nu_{P=O}$ 1268–1273 |
| IR bands ($cm^{-1}$) | The main groups IR bands ($cm^{-1}$) of compounds in $H_2O$ fraction |
| $\nu_{C=O}$ sh 1705–1720 | **organic acids** – acids of citric cycle, phenylpropanoids, oxalic, amino acids and other plant organic acids, $\nu_{C=O}$ sh 1720–1725 probably dimeric form |
| $\nu_{COO^-}$ sh 1600–1627 | asymmetric $\nu_{COO^-}$; **Salts of organic acids** (acids of citric cycle, phenylpropanoid, oxalic, amino acids and other plant organic acids) and interconnected bands: symmetric $\nu_{COO^-}$ 1385–1405 (sh 1405) |
| $\nu_{C=O}$ sh 1665–1680 | amides – band of $\nu_{C=O}$ is characterized as Amide I – **oligopeptides**: for example phytochelatins, glutathione |
| $\nu_{AROM.}$ 1510–1515 | secondary metabolism of polar compounds of phenylpropanoid metabolism (for example **polar flavonoid glycosides**) and interconnected bands: $\nu_{C-O}$ 1070 (sh 1070), $\nu_{C-O}$ 1052–1054, $\nu_{C=O}$ 1620–1630 conjugated ketone (**flavonoids**), $\nu_{OH,NH}$ 3401–3417 |

sh – shoulder

Table 5. The relationship between isolated compounds of BuOH fraction of individual treatments and sampling periods characterised by coefficient of determination $R^2$ using linear ($R_1^2$) and polynomial function of the 2nd degree ($R_2^2$)

| Effect of | $R_1^2$ | $R_2^2$ | Effect of | $R_1^2$ | $R_2^2$ |
|---|---|---|---|---|---|
| CK-AE on the CK-F | 0.993 | 1.000 | CK-F on the CK-AE | 0.993 | 1.000 |
| | 0.980 | 1.000 | | 0.961 | 0.999 |
| CK-AE on the GS-SG | 0.688 | 0.788 | GS-SG on the CK-AE | 0.688 | 0.733 |
| | 0.684 | 0.987 | | 0.684 | 0.975 |
| CK-F on the GS-SG | 0.723 | 0.791 | GS-SG on the CK-F | 0.723 | 0.793 |
| | 0.547 | 0.995 | | 0.547 | 0.961 |
| ES-PPhL on the CK-AE | 0.882 | 0.998 | CK-AE on the ES-PPhL | 0.882 | 0.985 |
| | 0.733 | 0.998 | | 0.733 | 0.997 |
| ES-PPhL on the AS-F | 0.651 | 0.653 | AS-F on the ES-PPhL | 0.651 | 0.828 |
| | 0.001 | 1.000 | | 0.001 | 0.675 |
| ES-PPhL on the GS-SG | 0.889 | 0.906 | GS-SG on the ES-PPhL | 0.889 | 0.995 |
| | 0.914 | 0.998 | | 0.914 | 1.000 |
| ES-PPhL on the CK-AE | 0.947 | 0.998 | CK-AE on the ES-PPhL | 0.947 | 0.992 |
| | 0.824 | 0.999 | | 0.824 | 0.999 |

CK-AE – conjugated ketone – typical for analogues of ecdysteroids (band: $\nu_{C=O}$ 1652/cm); CK-F – conjugated ketone–flavonoids (bands: $\nu_{C=O}$ 1622–1623/cm); GS-SG – glycosyl substances–steryl glycosides (bands: $\nu_{C-O}$ 1070–1071/ cm); AS-F – aromatic substances–flavonoids (bands: $\nu_{AROM}$ 1513–1515/cm); ES-PPhL – ester substances–polar phospholipids (band: $_{C=O}$ 1726–1733/cm)

The results confirmed the effect of more factors (not only N nutrition) on biosyntheses of isolated compounds.

Analyses of individual variants treated with nitrogen showed that metabolism of phospholipids and steryl glycosides contained in BuOH

Table 6. The relationship between isolated compounds of $H_2O$ fraction of individual treatments and sampling periods characterised by coefficient of determination $R^2$ using linear ($R_1^2$) and polynomial function of the 2nd degree ($R_2^2$)

| Effect of | $R_1^2$ | $R_2^2$ | Effect of | $R_1^2$ | $R_2^2$ |
|---|---|---|---|---|---|
| OA on the amides | 0.197 | 0.468 | amides on the OA | 0.197 | 0.952 |
| | 0.632 | 0.677 | | 0.632 | 0.984 |
| OA on the CK-FG | 0.093 | 0.743 | CK-FG on the OA | 0.093 | 0.856 |
| | 0.775 | 0.824 | | 0.775 | 0.989 |
| OA on the SOA | 0.057 | 0.967 | SOA on the OA | 0.057 | 0.401 |
| | 0.617 | 0.643 | | 0.617 | 0.906 |
| Amides on the CK-FG | 0.855 | 0.856 | CK-FG on the amides | 0.855 | 0.941 |
| | 0.971 | 0.991 | | 0.971 | 0.998 |
| Amides on the SOA | 0.221 | 0.283 | SOA on the amides | 0.221 | 0.417 |
| | 0.997 | 0.997 | | 0.997 | 0.997 |
| CK-FG on the SOA | 0.562 | 0.626 | SOA on the CK-FG | 0.562 | 0.755 |
| | 0.956 | 0.990 | | 0.956 | 0.967 |

OA – organic acids (bands: $\nu_{C=O}$ 1705–1725/cm); amides (bands: $\nu_{Amide\ I}$ 1665–1680/cm); AS-FG–aromatic substances–flavonoid glycosides (bands: $\nu_{AROM}$ 1510–1515/cm); SOA – salts of organic acids (symmetric $\nu_{COO}$– 1385–1405/ cm), including oxalate (band: $\nu_{COO}$- 1385/cm); CK-FG – conjugated ketone–flavonoid glycosides (bands: $\nu_{C=O}$ 1620–1630/cm)

fraction is connected with regulation of ecdysteroids metabolism. Metabolism of ecdysteroids is also connected with metabolism of flavonoids – compounds protected the cells against oxidative stress. Liu et al. (2010) and Rubin et al. (2009) confirmed the influence of nitrogen nutrition on the secondary metabolism and synthesis of flavonoids. According to our results and according to Pavlík et al. (2010b) the role of ecdysteroids in plants is connected with function of flavonoids, for example protection against oxidative stress. The relation between polar phospholipids (probably double bonds of unsaturated fatty acids) and flavonoid compounds was not confirmed.

The relative contents of compounds isolated from plants growing after application of 2 g N into water fraction were affected by sampling period (Table 6). The effect of nitrogen form was not observed. We can see the relationship mainly between amide substances of oligopeptides (for example glutathione – band amide I 1665–1680/ cm) and bands of organic acids and their salts (1385–1405/ cm). The correlation was calculated for typical band (1385/cm) for oxalates and bands characterizing amides, polar metabolites of phenylpropanoid metabolism and organic acids. Linear dependences of relative contents of organic acids and amides ($R^2$ 0.20) or organic acids and very polar flavonoid glycosides ($R^2$ 0.09) or organic acids and their salts ($R^2$ 0.06) or amides and salts of organic acids ($R^2$ 0.22) were low. After application of 4 g N linear dependences were increased ($R^2$ 0.63, 0.78, 0.62 and 1.00). For 4 g N treatments we can observe especially the effects of N nutrition. Both N rates affected linear correlations of relative contents of amides and polar flavonoid glycosides ($R^2$ 0.86 and 0.97) or polar flavonoid glycosides and salts of organic acids ($R^2$ 0.56 and 0.96). Biosyntheses of compounds contained in water fraction were affected only by one basic factor, or by major gene. This finding resulted in the fact that the calculation of polynomial function of the 2$^{nd}$ degree showed mostly similar dependences. The linear correlations were not close therefore we can suppose the effect of minor genes. These genes have a minor effect on biosyntheses of isolated compounds and this effect is typical for polygenic trait of phenotypic or genotypic variance.

Differences of individual experimental treatments showed the effect of nitrogen nutrition on significant relation between metabolism of oligopeptides and polar substances of phenylpropanoid metabolism (for example polar flavonoid glycosides) and also correlation amides and oxalates (Table 6). Werner and Schmidt (2002) described the formation of ammonia salts of organic acids in plants. According to Morita et al. (2004) ammonium ions induce formation of oxalic acid and its ammonium salt from ascorbic acid and simultaneously increase the activity of oxalate oxide. Oxalate oxide participates in developmental processes by producing $H_2O_2$, which is involved in the oxidative cross-linking of cell wall polymer (Morita et al. 2004) in addition to protecting against fungal infection (Bolwell and Wojtaszek 1997) and producing pathogenesis-related protein (Chamnongpol et al. 1998). These results confirmed that oxidative stress is affected by nitrogen application by the CULTAN method. The plants responded to stress by formation of salts (mainly salts of oxalic acid) and polar substances of phenylpropanoid metabolism (for example flavonoids). This response is confirmed by strong dependence of amide substances and flavonoids and also organic acids (including their salts) and flavonoids.

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