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Variation of antioxidants and secondary metabolites in nitrogen-deficient barley plants

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ABSTRACT

Barley (*Hordeum vulgare* cv. Bojos) plants cultured in low nitrogen (N) containing Hoagland solution (20 mg/l) were exposed to N deficiency (−N) over 15 days. Plants revealed relatively high tolerance to total N deficit because shoot length was not altered and dry biomass was depleted by ca. 30% while root length increased by ca. 50% and dry biomass remained unaffected. Soluble proteins and free amino acids decreased more pronouncedly in the roots. Antioxidants (glutathione and ascorbic acid) decreased in the shoots but increased or were not affected in the roots. Ascorbate peroxidase and glutathione reductase activities were depleted in shoots and/or roots while guaiacol peroxidase activity was stimulated in the shoots. In accordance, fluorescence signal of reactive oxygen species (ROS) and nitric oxide was elevated in shoots but no extensive changes were observed in roots if +N and −N treatments are compared. At the level of phenolic metabolites, slight increase in soluble phenols and some phenolic acids and strong elevation of flavonoid homoorientin was found in the shoots but not in the roots. Fluorescence microscopy in terms of detection of phenols is also discussed. We also briefly discussed accuracy of quantification of some parameters owing to discrepancies in the literature. It is concluded that N deficiency induces increase in shoot phenolics but also elevates symptoms of oxidative stress while increase in root antioxidants probably contributes to ROS homeostasis aimed to maintain root development.

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Introduction

Nitrogen (N) is essential plant macronutrient because it is involved in the biosynthesis of amino acids, proteins and enzymes (Scheible et al., 2004; Kováčik and Bačkor, 2007). Owing to N importance in metabolism, their limited availability or deficiency results in reduced growth and lower yield of plants (Rubio-Wilhelmi et al., 2011). At the same time, shift from N-based to C-based compounds is usually observed (Rubio-Wilhelmi et al., 2012a,b). These C-based metabolites include mainly phenolics, such as phenolic acids, flavonoids as well as coumarins (Kováčik et al., 2007; Giorgi et al., 2009; Rubio-Wilhelmi et al., 2012a). This effect of N deficiency on phenolic content is typical because depletion of other macronutrient such as potassium does not elevate phenols (Nguyen et al., 2010) while phosphate deficiency symptoms are partially similar to those of N deficit (Juszczuk et al., 2004).

Phenolics (or part of phenolics) are important so-called non-enzymatic antioxidants and include several thousands of compounds. They act as efficient antioxidants both in plants and in human diet, therefore studies of their accumulation in crop/medicinal plants received greater attention owing to produce more healthy preparations (Giorgi et al., 2009). Owing to direct connection between C and N metabolism, manipulating the N level is a good tool how to increase amount of phenols in plants (Kováčik et al., 2011). Besides, such studies are also important in terms of more complex responses of metabolites and metabolism within plant tissue, providing information about stress tolerance.

Environmental stress typically stimulates enhanced production of reactive oxygen species (ROS) in aerobic organisms including plants (Noctor and Foyer, 1998). Nitric oxide (NO) is another important gaseous molecule being involved in the regulation of metabolism (Kováčik et al., 2010). These molecules are also formed under N deficiency (Kováčik et al., 2009) or potassium deficiency (Hernandez et al., 2012). ROS overproduction is therefore controlled by various non-enzymatic (phenolics, glutathione, ascorbic acid) and enzymatic (ascorbate peroxidase, guaiacol peroxidase, glutathione reductase) antioxidants (Sakihama et al., 2002; Chen et al., 2010; Gajewska and Skłodowska, 2010).

Barley (*Hordeum vulgare*) is an important crop species for foodstuff and beer brewing industry and involves numerous cultivars (Dvořáková et al., 2008). Its responses to environmental stress were tested mainly after application of heavy metals (Chen et al., 2010). Known metabolic profile involves phenolics in caryopsis (Dvořáková et al., 2008) or excellently identified flavones in leaf

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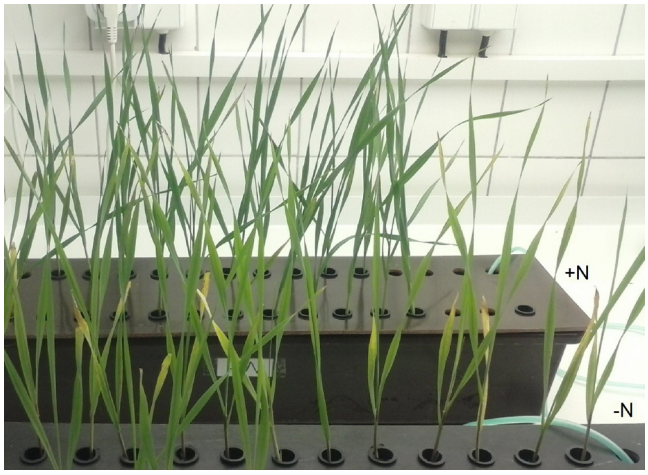

Fig. 1. Representative photo of *Hordeum vulgare* cultivation in hydroponics. Note slight chlorosis of N-deficient (−N) plants in comparison with control (+N) after 15 days of N deficiency.

biomass (Ferreres et al., 2008). Surprisingly, basic antioxidants such as ascorbate and glutathione involve such variable values in leaf and/or root tissue that they could only hardly be ascribed to various cultivars (Palatnik et al., 2002; Finkemeier et al., 2003; Chen et al., 2010).

Although manipulation of nitrate availability was intensively studied in various plants (see above), alteration of metabolism in terms of N nutrition in barley is only poorly known (Finkemeier et al., 2003). Quantitative data are completely missing in terms of secondary metabolites in vegetative organs. We therefore complexly studied response of known Czech barley cultivar (cv. Bojos) to nitrate deficiency over sufficiently long exposure period until appearance of visible symptoms. Growth parameters, amino acids, phenolics and other antioxidants including selected enzymatic activities were monitored and are complexly discussed in relation to growth changes. Extensive fluorescence microscopy was also performed to allow visualization of some parameters. Many parameters in barley plants are presented here for the first time and responses to N deficiency are therefore compared with other plant species or other stress impacts. We also briefly discussed accuracy of quantification of some parameters measured in barley owing to discrepancies in the literature.

Materials and methods

Plant culture, experimental design and statistics

Caryopses (grains) of barley (*Hordeum vulgare* cv. Bojos) were surface-sterilized with 70% ethanol for 1 min, rinsed with deionised water and placed on Petri dishes with filter paper and deionised water. After 48 h, homogenous germinated seedlings were placed to 1/10-strength Hoagland solution (containing 403 μM $Ca(NO_3)_2{\cdot}4H_2O$, 52.2 μM $NH_4H_2PO_4$, 604 μM KNO_3, 199 μM $MgSO_4{\cdot}7H_2O$, 35.6 μM NaOH, 28.8 μM KOH, 8.92 μM EDTA, 8.96 μM $FeSO_4{\cdot}7H_2O$, 9.68 μM H_3BO_3, 2.03 μM $MnCl_2{\cdot}4H_2O$, 0.314 μM $ZnSO_4{\cdot}7H_2O$, 0.210 μM $CuSO_4{\cdot}5H_2O$, 0.139 μM Na_2MoO_4 and 0.0859 μM $CoCl_2{\cdot}6H_2O$, pH maintained at 6.0) in 7-L brown plastic pots with continual aeration (Fig. 1). One pot contained 25 seedlings and solutions were changes every 5 days. Whole cultivation was performed in a growth chamber under controlled conditions: 12 h day (6.00 am to 6.00 pm); photon flux density was ~250 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes L36W/840 (Lumilux, Osram, Germany); 25/20 °C day/night temperature; and relative humidity ~60%. After 15 days of cultivation, part of plants was subjected to nitrogen (N) deficiency by substituting N-containing salts with equimolar salts without N (Kováčik and Bačkor, 2007; Kováčik et al., 2011). Control plants were further cultured in N-containing medium. Because first symptoms of N deficiency on shoots appeared after 10–11 days, experiment was closed after 15 days of N-deficient conditions. Then plants were harvested and separated to shoots and roots and length, fresh and dry mass were measured. For parameters measured in fresh samples, whole shoots or roots were powdered using liquid N_2 and assayed as described below. Spectrophotometry was carried out with Agilent/HP DAD UV/Vis 8453 Spectrophotometer. Fluorescence microscopy was done with Axioscop 40 microscope (Carl Zeiss, Germany) equipped with appropriate set of excitation/emission filters.

Data were evaluated using Student's *t*-test by comparison control (+N) and nitrogen-deficient (−N) variant for each parameter. Number of replications (*n*) in tables/figures denotes individual plants measured for each parameter. Two independent repetitions of the whole experiment were performed in order to check reproducibility.

Measurement of growth, tissue water content and nitrogenous metabolites

Fresh and dry matters were measured in order to determine the plant water content [100 − (dry mass × 100/fresh mass)] allowing recalculation of parameters measured in fresh samples. These dried samples were ground to a fine powder and analyzed for free amino acids and phenolics.

Soluble proteins were quantified according to Bradford method (1976) in homogenates prepared using 50 mM potassium phosphate buffer containing 5 mM insoluble PVPP (pH 7.0, 1 g FW/5 ml) and bovine serum albumin as standard. Free amino acids were extracted with 80% aqueous ethanol using computer controlled IKA Werke 50 device related to Soxhlet apparatus and analyses were performed on an HP 1100 liquid chromatograph (Hewlett Packard, Waldbronn, Germany) with fluorometric detector FLD HP 1100 and using precolumn derivatization with *o*-phtalaldehyde and 9-fluorenylmethyl chloroformate (Kováčik et al., 2011).

Quantification of glutathione, ascorbic acid and antioxidative enzymes

Reduced (GSH) and oxidized glutathione (GSSG) and ascorbic acid (AsA) were extracted with 35 mM HCl (0.2 g FW/2 ml) and quantified using LC–MS/MS (Agilent 1200 Series Rapid Resolution LC system coupled on-line to a detector) Agilent 6460 Triple quadrupole with Agilent Jet Stream Technologies (Kováčik et al., 2012) at *m/z* values 308/76, 613/231 (Airaki et al., 2011) and 177/95 in positive MRM mode, respectively. Separation was done using column Zorbax SB-C18 50 × 2.1 mm, 1.8 μm particle size and mobile phase consisting of 0.2% acetic acid and methanol (95:5). The flow-rate was 0.6 ml/min and column temperature was set at 25 °C. Freshly prepared standards were used for calibration and quantification.

Activities of antioxidative enzymes were measured in potassium phosphate buffer homogenates prepared as mentioned above. Ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) activities were measured as the oxidation of ascorbate (290 nm) and guaiacol (470 nm) and the reduction of GSSG (412 nm), respectively (Kováčik and Bačkor, 2007; Kováčik et al., 2009).

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