

Phenylalanine ammonia-lyase activity and phenolic compounds accumulation in nitrogen-deficient *Matricaria chamomilla* leaf rosettes

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Abstract

Phenylalanine ammonia-lyase (PAL) activity, accumulation of 11 phenolic acids and 3 coumarin-related compounds (herniarin and its glucosidic precursors (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acids; umbelliferone), biomass production, plant water content and lipid peroxidation status of *Matricaria chamomilla* L. leaf rosettes subjected to nitrogen (N) deficiency over 12 days were studied. Both biomass and water content of N-deficient rosettes decreased in comparison to controls, while quantities of most benzoic acids (*p*-hydroxybenzoic, syringic, vanillic), cinnamic acids (caffeic, chlorogenic, *o*- and *p*-coumaric, ferulic), herniarin and (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acids increased with prolonged nitrogen deficiency. However, three benzoic derivatives (gallic acid, protocatechuic acid and protocatechuicaldehyde) did not differ significantly from the controls. The content of umbelliferone, a stress metabolite in the leaves of chamomile, was not affected by N deficiency. It thus appears that synthesis of benzoic and cinnamic acids and of coumarins is probably regulated independently. PAL activity was significantly higher in N-deficient leaf rosettes after 4 and 8 days (by 61 and 23%, respectively), but decreased to control level by the end of the experiment. Nevertheless, it can be concluded that this is important biochemical factor contributing to the observed increase of phenolic compounds accumulation by producing nitrogen-free skeletons of *t*-cinnamate for subsequent pathways of phenylpropanoid metabolism. We also observed that the mechanism of nitrogen deficiency tolerance due to enhanced PAL activity is temporally limited. The non-altered extent of lipid peroxidation expressed as malondialdehyde amounts revealed that membrane integrity was not affected by N deficiency. This phenomenon in correlation to the antioxidative properties of phenolic metabolites is also discussed.

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1. Introduction

Optimal mineral nutrition is fundamental to the growth and productivity of plants and the macronutrient nitrogen (N), which is essential for amino acids, proteins and enzymes biosynthesis, represents quantitatively the most important element [1]. Its deficiency can be considered an abiotic stress factor, resulting, for example, in increased H₂O₂ production in *Arabidopsis* roots [2]. One of the general aspects of nitrogen deficiency is reduced root hydraulic conductivity making leaves unable to maintain adequate turgor [3].

Low nitrogen typically results in accumulation of phenolics [4–6], such as flavonols [7], anthocyanins [8] and coumarins [9]. Phenolics are the most widely distributed secondary plant products (30–45% of plant organic matter [10]) and are derived mainly from L-phenylalanine via nitrogen-free skeletons of *t*-cinnamate [10,11]. This initial step of phenylpropanoid synthesis is mediated by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and its activity under conditions of decreased nitrogen concentration has been inferred from changes in metabolic products [7,8] or levels of mRNA transcripts [6,12]. Under conditions of decreased nitrogen availability, plant phenolic content and PAL activity may increase because of decreased demand for proteins involved in growth [13,14]. Ammonium ions released by PAL can be assimilated, for example, via the GS/GOGAT system [10] and the resulting nitrogen-free carbon skeletons of *t*-cinnamate can be shunted into different phenylpropanoid pathways [7,8].

Abbreviations: d.m., dry mass; f.m., fresh mass; GMCAs, 2- β -D-glucopyranosyloxy-4-methoxycinnamic acids; MDA, malondialdehyde; PAL, phenylalanine ammonia-lyase; ROS, reactive oxygen species

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Benzoic and cinnamic acid derivatives (so-called phenolic acids) are universal phenolics in higher plants (e.g. caffeic, *p*-coumaric and ferulic acid); *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids occur in angiosperms [11]. The higher accumulation of *p*-coumaric and ferulic acid related to N deficiency has been described in leaves of *Oryza sativa* [5] and higher accumulation of chlorogenic, *p*-coumaric and caffeic acid in N-deficient *Nicotiana tabacum* plants, while ferulic acid remained unaltered or decreased [6]. On the other hand, ferulic acid increased in *Arabidopsis* plants [12]. In *N. tabacum*, nitrogen starvation increased the content of chlorogenic acid four-fold and stronger lignification of the stem occurred [6].

Coumarins are lactones of *o*-hydroxycinnamic acids with limited distribution within the plant kingdom [11]. In chamomile, herniarin is the most abundant coumarin derivative together with its native glucosidic precursors (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs) ([15,16] and the references therein). Umbelliferone, considered a stress coumarin of chamomile, is present in very low amounts under non-stress conditions, with its content increasing after abiotic stress such as foliar Cu^{2+} application or biotic stress [15]. These stress influences cause increases in herniarin and decreases in GMCAs [15,16]. This correlation has been noted also in the leaves of chamomile plants after hydroponic application of salicylic acid [17]. However, the same response was not observed in N-deficient chamomile plants, indicating another explanation for accumulation of coumarins, which negatively correlated with aromatic amino acids contents [9]. Additionally, cadmium [18] and copper (Kováčik, unpublished data) application into the hydroponics did not cause “typical” stress response of coumarins accumulation compared to previous papers [15–17].

Environmental stresses such as nitrogen deficiency [2] and the presence of heavy metals [19] stimulate formation of reactive oxygen species (ROS), which can damage biomolecules including membrane lipids [20]. Malondialdehyde (MDA) is a marker commonly used for assessing of membrane lipid peroxidation. In plants, tissue damage occurs when the capacity of their antioxidative systems is insufficient to counteract the amount of ROS being generated [21]. Phenolic compounds are important antioxidants; for example, inhibition of chlorogenic acid synthesis leads to increased levels of MDA [22]. In terms of plant defence, nitrogen deficiency-induced accumulation of phenolics may afford protection against further sources of stress such as pathogen attack or light-induced damage [7].

The main aim of our study was to determine: (i) the influence of nitrogen deficiency on biomass accumulation and plant water content, (ii) changes in PAL activity related to prolonged nitrogen deficiency, (iii) quantities of phenolic acids and coumarin-related compounds during the course of nitrogen deficiency and (iv) whether, based on MDA accumulation, nitrogen deficiency causes membrane damage.

2. Materials and methods

2.1. Cultivation, plant dry mass and water content determination

Twenty-one-day old plants of *Matricaria chamomilla* L. (tetraploid ‘Lutea’, Asteraceae) germinated in sand were transplanted into Hoagland solution [23] with two equimolar modifications of micronutrients (Na_2MoO_4 instead of MoO_3 and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ instead of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) routinely used in our laboratory [9,18]. Ten uniform plants per litre were cultivated in brown plastic 7 L boxes (70 plants/box) with continual aeration of solutions. The experiment was performed in a growth chamber under controlled conditions: 12 h day (6:00 a.m.–6:00 p.m.), with photon flux density of $210 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at leaf level supplied by cool white fluorescent tubes TLD 36W/33 (Philips, France), 25/20 °C day/night temperature and relative humidity 60%. Solutions were renewed weekly to prevent nutrient depletion [23]. Plants which had been cultivated in hydroponics for 3 weeks were used in the experiment and further cultured either in a nitrogen-deficient medium in which $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ was replaced with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{NH}_4\text{H}_2\text{PO}_4$ with KH_2PO_4 and KNO_3 with K_2SO_4 , or in a complete nutrient solution [9]. Nitrogen-deficient and control solutions were renewed on the seventh day after the start of nitrogen deficiency. Control and N-deficient leaf rosettes were collected mid-day at 96 h intervals over 12 days of the experiment. Fresh and dry masses were estimated in order to determine the plant water content [$100 - (\text{dry mass} \times 100 / \text{fresh mass})$]. Samples were dried at 95 °C, homogenized in a blender and stored in a desiccator until analysed. Fresh samples for measurement of phenylalanine ammonia-lyase activity and malondialdehyde content were immediately analysed.

2.2. Phenylalanine ammonia-lyase (PAL) activity

PAL was extracted and its activity was determined using the HPLC method [24,25]. A lower final volume of reaction mixture (1.1 mL) was used to improve detection of the PAL reaction product, *trans*-cinnamic acid (*t*-CA). Whole fresh rosettes were ground at 4 °C in 0.1 M sodium borate buffer (pH 8.8), 2 g plant material 5 mL^{-1} of buffer. Homogenates were centrifuged (Boeco U-32R, Boeckel & Co., Hamburg, Germany) at 4 °C ($12,000 \times g$ for 15 min) and the supernatant was used as the enzyme extract. Reaction mixtures consisting of 500 μL sodium borate buffer (pH 8.7) and 250 μL enzyme extracts were pre-incubated at 40 °C (5 min) and the reaction was started by adding 300 μL 50 mM L-phenylalanine (Sigma–Aldrich, Germany). After 1 h incubation at 40 °C, the reaction was stopped by adding 50 μL 5 N HCl. The reaction mixture was centrifuged again ($12,000 \times g$ for 15 min) prior to injection into the HPLC at room temperature. The HPLC system consisted of an Ecom pump (Prague, Czech Republic), a Rheodyne injector valve (20 μL), a Hewlett-Packard UV–vis variable detector model 1050 (HP, Palo Alto, CA, USA), a SGX C 18 (7 μm) column (3 mm $\times$ 150 mm) and an Apex integrator (Prague, Czech Republic). The mobile phase was a 57% acetonitrile with flow rate of 0.5 mL min^{-1} . All

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