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Effect of nitrogen starvation on the phenolic metabolism and antioxidant properties of yarrow (*Achillea collina* Becker ex Rchb.)

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ABSTRACT

In this study, the effects of long-term nitrogen deficiency (N 0.1 mM for 4 months) on growth, phenolic content and activity of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) were investigated in the leaves, inflorescences and roots of yarrow (*Achillea collina* Becker ex Rchb.) grown in hydroponics. The antioxidant capacity of methanol extracts was also evaluated. Nitrogen starvation decreased plant growth and the leaves' total nitrogen, amino acids, proteins, chlorophylls and carotenoids contents indicating that the primary metabolism was severely limited by low nitrogen availability. The amount of total phenolics and the antioxidant capacity were higher in leaves and roots of nitrogen-starved compared to control plants. The most abundant phenolic acids identified in yarrow were 5-*O*-caffeoylquinic acid (chlorogenic acid), and 3,5 and 4,5-di-*O*-caffeoylquinic acids. Nitrogen starvation significantly increased the contents of all these compounds and the PAL activity in leaf and root tissues. Results suggest that hydroponics, with proper manipulation of nutritional factors, might be a suitable system for the production of compounds with health benefits, such as caffeic acid derivatives, in yarrow.

1. Introduction

Achillea collina Becker ex Rchb. (yarrow) is a tetraploid proazulenes-containing species of the *Achillea millefolium* aggregate cultivated in European alpine areas. The above-ground parts of yarrow are commonly used in traditional medicine, both internally, as infusions, and in alcoholic beverages for their digestive, anti-inflammatory, analgesic and antipyretic activities and, externally, to treat skin inflammations and for wound healing (Willuhn, 2002). Beside the sesquiterpenes, which have been considered to be mostly responsible for the pharmacological activity of plant extracts, there is increasing evidence that phenolic compounds represent another potentially health-relevant group of secondary metabolites in yarrow (Benedek, Gjoncaj, Saukel, & Kopp, 2007). In fact, the flavonoids from yarrow mediate spasmolytic activity (Lemmens-Gruber et al., 2006), whereas the choleretic effects are caused by the dicaffeoylquinic acids (Benedek, Geisz, Jager, Thalhammer, & Kopp, 2006).

Phenolic compounds represent the largest group of plant secondary metabolites that embrace a variety of structural classes (e.g. as precursors of lignin) and biological functions. They originate from *trans*-cinnamic acid, which is produced by the action of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), the key enzyme of the phenylpropanoids pathway. An increase in phenolic compound biosynthesis and accumulation frequently occurs in plant tissues as a reaction to biotic and abiotic stresses (Dixon & Paiva, 1995). Among abiotic stresses, mineral nutrient starvation has been reported to affect phenylpropanoid metabolism. In particular, in nitrogen deficiency conditions, there was an accumulation of phenolic compounds, such as flavonols (Stewart et al., 2001), anthocyanins (Bongue-Bartelsman & Phillips, 1995) and phenolic acids, including benzoic, cinnamic and coumarin derivatives (Kováčik, Klejdus, Bačkor, & Repčák, 2007).

It is known that environmental stresses, including nitrogen starvation (Kováčik & Bačkor, 2007), may increase the production of reactive oxygen species (ROS). Plants have evolved a complex array of non-enzymatic and enzymatic detoxification mechanisms to protect themselves from oxidative damage. In this area, phenolic compounds may play an important role as scavengers of free radicals and other oxidative species (Grace & Logan, 2000; Rice-Evans, Miller, & Paganga, 1997).

In the past few years, interest in the antioxidant properties of plant-derived foods and medicinal plants has increased, since antioxidants contained in plants are involved in the preservation of human health. Moreover, plant extracts with antioxidant properties are becoming more and more attractive for the food industry, as they are considered to represent a "natural" alternative to synthetic antioxidants. Hence, the assessment of antioxidant properties of traditional medicinal plants, which are widely used, is an

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important concern in the quest for new sources of natural antioxidants.

The consumption of herbal medicines is widespread and is continuously increasing worldwide. Medicinal plants are usually collected from the wild, leading to the loss of genetic diversity and habitat devastation, and making the quality of the herbal extracts unpredictable, due to genetic/environmental factors and the potential presence of contaminants (Canter, Thomas, & Ernst 2005). Growing genetically homogeneous populations of medicinal plants in controlled environments would overcome these problems and may have the potential to increase secondary metabolite yield. Hydroponic systems offer the advantages of easy management of nutritional and environmental parameters which are closely involved in secondary metabolite biosynthesis. In fact, appropriate manipulation of the nutritive solution composition and the growth system has been effective in enhancing secondary metabolite yield in a variety of medicinal plant species (Montanari et al., 2008; Zheng, Dixon, & Saxena, 2006).

Therefore, the purposes of this research were to evaluate the effects of long-term nitrogen deficiency on biomass accumulation, phenylpropanoids metabolism and PAL activity in different organs (leaves, inflorescences and roots) of yarrow plants grown in hydroponics. In addition, the consequences of nitrogen starvation on photosynthetic pigments, total protein, total amino acid and total nitrogen in leaves, were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·), gallic acid, atropine, glutamic acid, caffeic acid and 5-*O*-caffeoylquinic acid (chlorogenic acid, CGA) were purchased from Sigma–Aldrich (Milan, Italy). All other chemicals and solvents used were of analytical grade.

2.2. Culture conditions and growth measurement

Two-month-old plants of yarrow (*A. collina* Becker ex Rchb. cv. "SPAK"), provided by Valplantons BIO (Saillon, CH), were transferred into polystyrene plugs floating in 3 l plastic pots filled with a nutrient solution containing 0.5 mM $Ca(NO_3)_2$, 0.1 mM $CaSO_4$, 0.15 mM K_2HPO_4, 0.3 mM K_2SO_4, 0.3 mM $MgSO_4$, 14 μM Fe–EDTA, 13.8 μM H_3BO_3, 2.7 μM $MnSO_4$, 0.24 μM $CuSO_4$, 0.09 μM $ZnSO_2$ and 0.03 μM Na_2MoO_4. The pH was adjusted to 6.2 with HCl (1–0.1 N). Ten homogeneous plants of the same clone were divided into two groups and cultured during a 4 month experiment, the first group in the complete nutrient solution (N 1 mM) and the second one in a nutritive solution in which $Ca(NO_3)_2$ was reduced by 10-fold (N 0.1 mM). In order to maintain the same Ca^{2+} concentration, 0.55 mM $CaSO_4$ was added to the N-deficient nutrient solution. The nutrient solutions were continuously aerated and renewed weekly. Experiments were performed in a greenhouse at a constant temperature of 25 °C, photoperiod of 16/8 h light/dark, with 200 μmol m^{-2} s^{-1} PAR supplied by halogen lamps (OSRAM Powerstar HQI–BT 400W), 400/700 nm, and 60% relative humidity.

To evaluate the effect of long-term nitrogen deficiency on yarrow growth, fresh weight of the whole control and N-deficient plants was periodically recorded during the experiments at 0, 13, 38 and 128 d. Roots were blotted dry with paper towels and the fresh weight was immediately recorded. In addition, fresh and dry weights of leaves, inflorescences, stems and roots of control and N-deficient plants were determined at the end of the experiments (128th d) when at least 80% of plants were in full bloom. Fresh samples were rapidly frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C. Dry weight was determined by drying part of the frozen sample in an oven at 60 °C to constant weight.

2.3. Determination of the total phenolic content

Phenolic compounds were extracted with methanol from frozen fresh and finely powdered samples of leaves, inflorescences and roots. Aliquots of each sample, after drying (20 mg), were mixed with 10 ml of methanol and kept in the dark for 24 h at room temperature. These extracts were then filtered through a 0.45 μm MILLEX® HV filter (Millipore, Milan, Italy), stored at −20 °C and used for both colorimetric assays and HPLC analysis of phenolic compounds.

The amount of total phenolics in the extracts was determined by the Folin–Ciocalteu method (Slinkard & Singleton, 1977), using gallic acid as standard. Distilled water (1.55 ml) was combined with 50 μl of sample, 100 μl of Folin–Ciocalteu's reagent and 300 μl of Na_2CO_3 (20%). The mixture was vortexed thoroughly and, after incubation at 40 °C for 30 min, the absorbance was measured at 765 nm against a blank without sample, using a UV/VIS spectrophotometer (Jasco–7800; Milan, Italy). Quantification was done on the basis of the standard curve of gallic acid (solution of gallic acid 20% ethanol, 0.25–5 μg ml^{-1}). Results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight.

2.4. Isolation of 3,5-di-O-caffeoylquinic acid (3,5-DCQA) and 4,5-di-O-caffeoylquinic acid (4,5-DCQA)

3,5-DCQA and 4,5-DCQA were isolated from the methanolic extract of yarrow by repeated preparative HPLC experiments using an AKTA Basic100 instrument (Pharmacia, Uppsala, Sweden). Chromatographic conditions were as follows: column, VP125/10 Nucleodur C18 Pyramid (10 μ, 125 × 10 mm, Macherey–Nagel); flow rate, 8 ml min^{-1}; detector, λ 340, 290, 254 nm; mobile phase, 0,05% formic acid in water, pH 2–3 (solvent A) and methanol (solvent B), gradient elution from 20% to 80%, solvent B, in 15 min; injection volume, 1 ml. Peaks with retention times of 5.0 and 5.9 min were collected and lyophilized, giving pure 3,5-DCQA (0.4% yield) and 4,5-DCQA (0.02% yield), respectively, which were identified by nuclear magnetic resonance (NMR) (Wu et al., 2007) and electrospray ionisation mass spectrometry (ESI-MS, ThermoFinnigan LCQ Advantage instrument, Hemel Hempstead, Hertfordshire, UK) (Clifford, Knight, & Kuhnert, 2005). NMR spectra were recorded on a Bruker AVANCE 400 spectrometer (Bruker, Karlsruhe, Germany) using a XWIN-NMR software package.

2.5. HPLC–MS analysis

HPLC-MS analyses were performed on a ThermoFinnigan LCQ Advantage instrument equipped with an electrospray ionisation source (ESI) operating in the negative mode. The HPLC system included a Surveyor LC pump and a Surveyor PDA Photodiode Array UV detector. MS operating conditions were as follows: capillary temperature, 275 °C; ionisation voltage, 3.5 kV; collision energy, 35%. Nitrogen was used as the sheath gas and the scan range was *m/z* 50–1000. Separations were achieved on a reversed phase column C-18 Hypersil ODS (Supelco, particle size 5 μm, 250 mm × 4.6 mm). Gradients were generated using 0.05% formic acid in water (solvent A) and methanol (solvent B) starting at 30% B at a constant flow rate of 1 ml min^{-1}. Solvent B was then increased to 70% at 5 min, 85% at 14 min, 100% at 16 min and finally, isocratic elution with 100% B until 20 min. The injection volume was 20 μl. Peaks corresponding to caffeic acid, CGA, 3,5-DCQA and 4,5-DCQA were identified by comparison of their retention times, UV and MS spectra with those of authentic reference compounds.

2.6. Quantitative analysis of phenolic compound

Two millilitres of the methanolic extracts were vacuum-dried overnight. Samples were re-dissolved in HPLC-grade methanol and properly diluted before injection (20 μl). Analyses of phenolic compounds was performed with an HPLC system consisting of a Waters 515 pump connected to a Waters 2487 Dual λ Absorbance UV Detector set at 290 and 340 nm (Waters, Vimodrone, MI, Italy). Chromatographic conditions were those described above for LC–MS analysis. Quantification of caffeic acid, CGA, 3,5-DCQA and 4,5-DCQA was achieved by external standardization with reference compounds. Data were processed with a computer-controlled system, using upgraded Millenium[32] software.

2.7. Antioxidant capacity assay

Antioxidant capacity (AC) was determined with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) according to the method described by Mensor et al. (2001). The same extracts as used for phenolic determination were diluted with methanol to 5 different increasing concentrations between 0.09 and 0.74 mg ml^{-1}. The DPPH· concentration in methanol was 0.1 g l^{-1}. One ml of extract solution (sample) or methanol (control) was mixed with 1 ml of DPPH· and the absorption was recorded after 20 min in the dark, at 517 nm. AC was calculated according to the following equation:

$$AC[\%] = 100 \times (A_C - A_S)/A_C$$

where A_s is the absorbance of the sample and A_c is the absorbance of the control. The concentration of extracts determining a 50% decrease of DPPH· solution absorbance (IC50), was calculated by linear regression of the concentration-response plots, where the abscissa represented the concentration (mg ml^{-1}) of the extracts and the ordinate the average percent of AC from three separate determinations. Results were then expressed as 1/IC50.

2.8. Determination of nitrogen and leaf nitrogen-related traits

For total nitrogen quantification, frozen leaf samples were dried in an oven at 60 °C to constant weight and 100 mg of each sample was then injected into a combustion elemental analyser (CE Instruments, NA 2100, Milan, Italy). Total nitrogen content was calculated using a standard curve made with increasing amount of atropine.

Free amino acids and total proteins were extracted from frozen fresh leaves (0.5 g) with 3 ml of 5% trichloroacetic acid (TCA). After centrifugation at 13,000*g* at 4 °C for 10 min, the supernatant was used for amino acids quantification according to Moore and Stein (1954), while the remaining pellets were analysed for total proteins, based on the method reported by Nieman & Poulsen (1963). The concentration of free amino acids was determined using the standard curve of glutamic acid (0.06–0.16 μg ml^{-1}). Total proteins were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard.

Chlorophylls and carotenoids were extracted from frozen fresh leaves (50 mg) with 10 ml of methanol for 24 h at 4 °C in the dark. The extracts were filtered through a 0.45 μm MILLEX® HV filter and the absorbance was measured at 470.0, 652.4 and 665.2 nm for quantification of carotenoids, chlorophylls a (Chl a) and chlorophylls b (Chl b), respectively. The amounts of carotenoids, Chl a, Chl b, Chl a+b were calculated as described by Lichtenthaler (1987), and expressed in mg g^{-1} dry weight.

2.9. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) extraction and assay

PAL extraction and assay were carried out as described by Yan, Shi, Ng, and Wu (2006), with minor modifications. Fresh frozen leaf, inflorescence and root samples (0.125 g) were homogenized for 5 min in a cold mortar with 1 ml of 100 mM phosphate buffer (pH 6), 2 mM EDTA, 4 mM dithiothreitol (DTT), and 20% (w/w) polyvinylpolypyrrolidone (PVPP). After centrifugation (13,500*g* for 25 min at 4 °C), the free-solid extracts (50 μl) were mixed with 0.5 ml 10 mM borate buffer (pH 8.8), 0.25 ml 20 mM L-phenylalanine (predissolved in 10 mM borate buffer pH 8.8) and 0.25 ml distilled water and incubated at 30 °C in a water bath for 4 h. PAL activity was determined by measuring the initial and final (4 h) absorbance of the reaction solution at 290 nm using a UV/VIS spectrophotometer (Cary Bio 50, Varian Italy). Absorbance of a blank without L-phenylalanine, but with the enzyme extract, was estimated, to avoid interference by endogenous L-phenylalanine. PAL activity was expressed as μmol of *trans*-cinnamic acid produced per h per mg of protein determined by the Bradford method (Bradford, 1976).

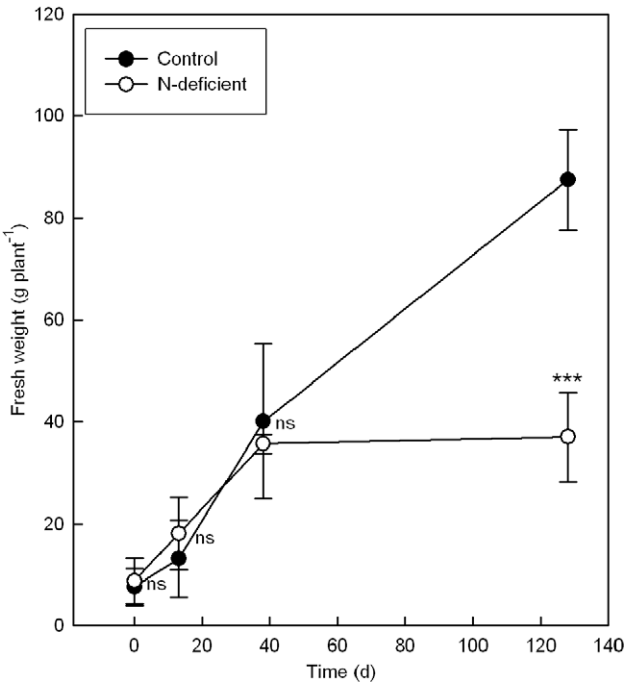

Fig. 1. Time-course of the whole plant fresh weight of control and nitrogen-starved (N-deficient) yarrow plants grown in hydroponics for 4 months. Each value is the mean ± SD ($n = 5$). For each sampling time (0, 13, 38 and 128 d), ns or *** indicate not significant or significantly different according to the Student's *t*-test at the $p < 0.001$ level.

Table 1
Dry weight (g) of different organs of control and nitrogen-starved (N-deficient) yarrow plants grown in hydroponics for 4 months

	Leaves	Inflorescences	Roots	Stems	Total biomass
Control	5.73 ± 0.9	1.06 ± 0.2	8.24 ± 1.6	4.93 ± 0.6	19.9 ± 2.0
N-deficient	1.56 ± 0.9***	0.37 ± 0.3**	3.85 ± 1.5**	1.51 ± 0.7***	6.9 ± 3.4***

Each value is the mean ± SD ($n = 5$). **, *** indicate significant differences according to the Student's *t*-test at the $p < 0.01$ and $p < 0.001$ levels, respectively.

2.10. Statistical analysis

The experiment was repeated at least three times. Since the results of each experiment were similar, statistical analysis was performed on the data recorded for the previous one. For all parameters, the Student's *t*-test was used to assess significant differences between control and N-deficient plants.

3. Results and discussion

3.1. Nitrogen starvation decreased plant growth and leaf nitrogen-related traits of yarrow plants

Nitrogen is an important inorganic nutrient for plants, being one of the major constituents of amino acids, proteins and nucleic acids. It is well known that nitrogen deficiency negatively affects plant growth and development and induces a wide reprogramming of primary and secondary metabolism (Scheible et al., 2004). In the present study, the level of nitrogen affected both fresh and dry weights of yarrow plants. During the first 40 d of culture, the increase of plant fresh weight was similar in nitrogen-starved and control plants but, afterwards, low nitrogen availability nearly inhibited plant development (Fig. 1). During this period, flower stalk formation and elongation occurred. Thus, at the end of the experiment (128th d), the dry mass of leaves, inflorescences, roots and stems was lower in the nitrogen-limited condition by 27%, 35%, 46% and 30%, respectively than in the control (Table 1). As a result, plant dry weight was about three times lower in nitrogen-starved than in control plants. The shoot/root ratio was higher in control (1.3) than in nitrogen-starved plants (0.8), indicating that, in nitrogen-limited conditions, photoassimilates were preferentially directed to root growth, allowing a more efficient nitrogen uptake from the nutritive solution (Scheible, Laurer, Schulze, Caboche, & Stitt, 1997).

Table 2 shows that, in leaves' total nitrogen, total amino acids and total protein contents were significantly lower in nitrogen-starved than in control plants (Table 2). In nitrogen-starved plants, the contents of chlorophyll a (Chl a), chlorophyll b (Chl b), chlorophyll a+b (Chl a+b) and total carotenoids were reduced compared to the control (Table 2). As previously reported by Scheible et al. (2004), our results show that the photosynthetic apparatus and the primary metabolism in yarrow plants were severely limited by low nitrogen availability.

3.2. Nitrogen starvation increased total phenolics accumulation and the antioxidant activity of yarrow extracts

Since several medicinal plants may contain substantial amounts of phenolics (e.g. Prakash, Suri, Upadhyay, & Singh, 2007) and exhibit considerable antioxidant activity (e.g. Miliauskas, Venskutonis, & van Beek, 2004), we assessed the total phenolics content and the DPPH· radical-scavenging activity of different organs of yarrow plants grown in hydroponics with full or reduced nitrogen levels (Fig. 2). The amount of total phenolics was signifi-

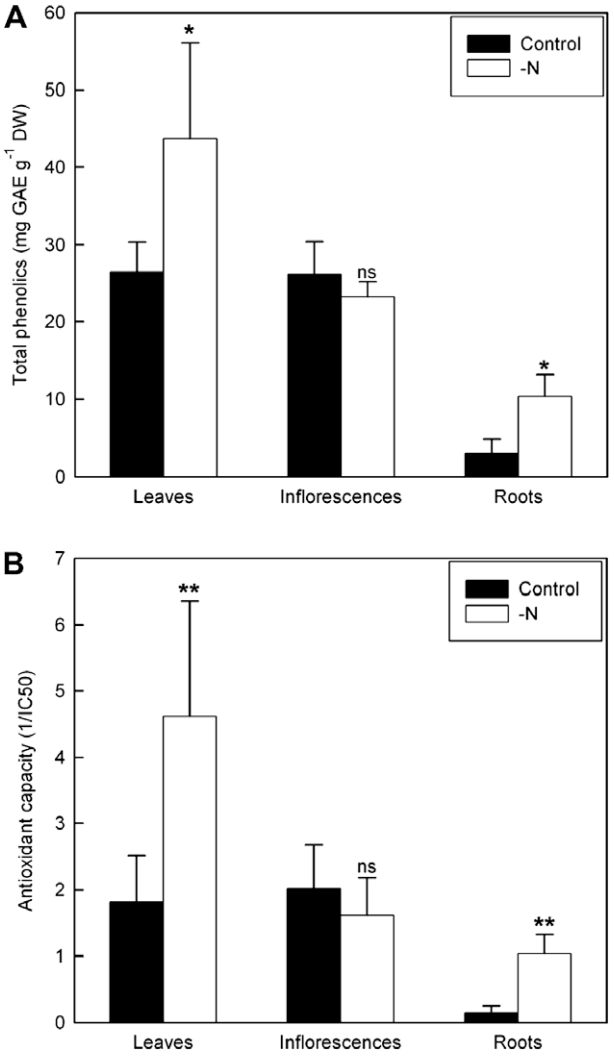

Fig. 2. Total phenolics content (A) and the antioxidant capacity (B), determined by the Folin–Ciocalteau and the DPPH. method, respectively, of different organs of control and nitrogen-starved (N-deficient) yarrow plants grown in hydroponics for 4 months. Each value is the mean ± SD ($n = 5$), ns, $_*$ and $_{**}$ indicate not significant or significantly different according to the Student's *t*-test at the $p < 0.05$ and $p < 0.01$ levels, respectively.

Table 2
Leaf nitrogen-related traits (mg g^{-1} DW) of control and nitrogen-starved (N-deficient) yarrow plants grown in hydroponics for 4 months

Leaf nitrogen-related traits	Control	N-deficient
Total nitrogen	36.8 ± 0.2	28.8 ± 0.2$_{***}$
Total amino acid	10.7 ± 1.2	5.98 ± 2.0$_{**}$
Total protein	34.7 ± 3.7	22.4 ± 7.3$_{*}$
Chlorophylls		
Chl a	7.30 ± 1.8	4.18 ± 1.1$_{*}$
Chl b	2.42 ± 0.6	1.40 ± 0.3$_{*}$
Chl a+b	9.71 ± 2.5	5.61 ± 1.4$_{*}$
Carotenoids	1.87 ± 0.5	1.16 ± 0.3$_{*}$

Each value is the mean ± SD ($n = 5$). ns, $_*$, $_{**}$, $_{***}$ indicate not significant or significantly different according to the Student's *t*-test at the $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively.

Table 3
Content of phenolic acids (mg g^{-1} DW) in different organs of control and nitrogen-starved (N-deficient) yarrow plants grown in hydroponics for 4 months

Phenolic acid		Leaves	Inflorescences	Roots
CGA	Control	2.33 ± 0.3	4.02 ± 1.7	0.56 ± 0.36
	N-deficient	5.58 ± 0.5$_{**}$	2.09 ± 0.3^{ns}	4.65 ± ± 0.8$_{***}$
Caffeic acid	Control	0.38 ± 0.2	ND	0.15 ± 0.1
	N-deficient	0.54 ± 0.3^{ns}	ND	ND
3,5-DCQA	Control	10.7 ± 4.2	10.56 ± 5.0	1.11 ± 1.2
	N-deficient	26.1 ± ± 11.3$_{*}$	6.84 ± 1.3^{ns}	8.98 ± 2.9$_{***}$
4,5-DCQA	Control	0.88 ± 0.24	2.10 ± 0.7	0.18 ± 0.1
	N-deficient	5.42 ± 0.9$_{**}$	1.31 ± 0.2^{ns}	1.06 ± 0.3$_{***}$

Each value is the mean ± SD ($n = 5$). ns, $_*$, $_{**}$, $_{***}$ indicate not significant or significantly different according to the Student's *t*-test at the $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively. ND means not detected.

cantly higher in leaves and roots of nitrogen-starved than in control plants (Fig. 2A). Analogous results were shown in *Matricaria chamomilla* L. (Kováčik & Bačkor, 2007) where a similar increase in the total phenolic content in leaves and roots of nitrogen-deficient plants was found. However, unlike the case mentioned above, we found that, in nitrogen-starved yarrow plants, the phenolic content in leaves was much higher than that in the roots. The leaf total phenolics content detected in this study was comparable to that found in yarrow plants cultured in open fields located in the Alps (Giorgi, Licheri, Mingozzi, & Cocucci, 2007). On the other hand, there were no differences between inflorescence extracts of control and nitrogen-starved plants, either in the total phenolic content (Fig. 2A) or in the level of specific phenolic acids (Table 3). Likewise, in field experiments we found that the total phenolic content

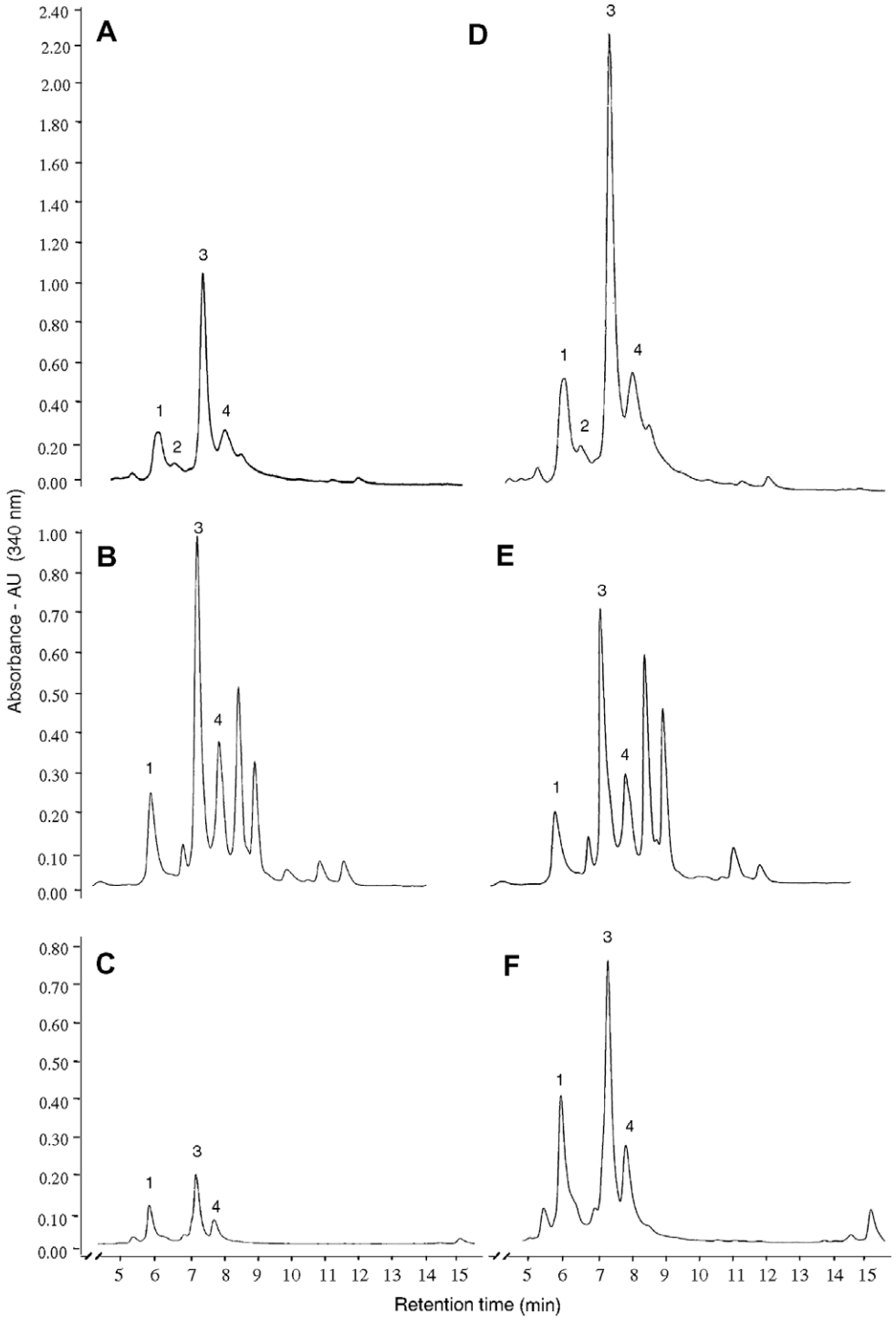

Fig. 3. HPLC profiles of leaf (A, D), inflorescence (B, E) and root (C, F) methanol extracts of control (A, B, C) and N-deficient (D, E, F) yarrow plants. Chromatograms are 5–15 min traces of maxplot detection at 340 nm. Column C-18 Hypersil ODS; mobile phase: 0.05% formic acid in water and methanol; flow rate: 1 ml min^{-1}; injection volume: 20 μl. Peak identification: 1, 5-*O*-caffeoylquinic acid (chlorogenic acid); 2, caffeic acid; 3, 3,5-di-*O*-caffeoylquinic acid (3,5-DCQA); 4, 4,5-di-*O*-caffeoylquinic acid (4,5-DCQA). Peaks without numbers have not been identified.

in inflorescences of plants cultivated under different coloured plastic films did not change significantly between treatments (Giorgi et al., unpublished results). Therefore, it appears that phenolic accumulation in inflorescences is an inherent characteristic of yarrow that is independent of the growth conditions applied (i.e. nitrogen level and light quality). This may also reflect distinct physiological roles of phenolic compounds in inflorescences and leaves.

Data on the antioxidant capacity of methanol extracts reflect the pattern seen for total phenolics (Fig. 2B). Indeed, in comparison with the control treatment, nitrogen starvation increased the antioxidant capacity of leaves and roots extracts by 39% and 135%, respectively (Fig. 2B). Inflorescence extracts had approximately the same antioxidant capacity in nitrogen-starved and control plants. A strong correlation ($r = 0.94$, $p < 0.001$) between the total phenolics content and the corresponding antioxidant capacity of methanol extracts was found. This relationship has been widely reported for several medicinal plant species (Miliauskas et al., 2004).

3.3. Nitrogen starvation induced the accumulation of specific phenolic acids and altered PAL activity

The HPLC profiles of leaf, inflorescence and root methanol extracts are shown in Fig. 3. As found by Benedek et al. (2007), the most abundant phenolic acids identified in yarrow were 5-*O*-caffeoylquinic acid (chlorogenic acid, CGA), 3,5-di-*O*-caffeoylquinic acid (3,5-DCQA) and 4,5-di-*O*-caffeoylquinic acid (4,5-DCQA). 3,5-DCQA and 4,5-DCQA were isolated from yarrow leaves by preparative HPLC. Their NMR (Wu et al., 2007) and negative ion electrospray (ESI) MS/MS spectra were found to be in agreement with those reported in the literature. Both gave a parent ion at *m/z* 515 $[M–H]^-$, and could be distinguished by their diagnostic patterns of fragmentation (Wu et al., 2007; Clifford et al., 2005).

Qualitative variations were observed among the different organs. Indeed, inflorescence and root extracts (Fig. 3B and C, respectively) displayed characteristic peaks that have not yet been identified. However, at least in inflorescence extracts, they might be flavonoids, as previously reported in flowering tops of yarrow (Benedek et al., 2007).

Furthermore, we found another phenolic acid, identified as caffeic acid. This compound was detected in small amounts in leaves of both nitrogen-starved and control plants, and in control plant roots, while it was totally lacking in inflorescences and in nitrogen-depleted roots (Table 3). Nevertheless, the level of nitrogen did not significantly affect caffeic acid accumulation. These results support the assumption that free acids rarely accumulate in plant cells but are usually conjugated to sugar, cell wall carbohydrates or organic acids (Dixon & Paiva, 1995).

For each organ, nitrogen starvation did not induce appreciable qualitative modification of the phenolic profiles when compared with the control treatment (Fig. 3). On the other hand, nitrogen starvation significantly altered phenolic acid levels in yarrow plants, and the accumulation pattern depended on the phenolic acid and the plant organ (Table 3). Compared to control plants, there were 2.5 and 8.5 increase in CGA content in leaves and roots of nitrogen-starved plants, respectively. Analogous results were reported in leaves of *M. chamomilla* (Kováčik et al., 2007) and tobacco plants (Fritz, Palacios-Rojas, Feil, & Stitt, 2006) cultured under nitrogen-deficient conditions. The amounts of CGA found in all yarrow samples are quite high compared to those in the same organs of other medicinal plants (Prakash et al., 2007). However, of particular relevance is the CGA content found in roots of nitrogen-stressed yarrow plants which is, to the best of our knowledge, the highest reported in the literature (Montanari et al., 2008). CGA is a taxonomically widespread phenylpropanoid metabolite which is emerging as an important dietary bioactive compound (Niggeweg, Michael, & Martin, 2004). Because of its antioxidant properties (Grace & Logan, 2000), CGA seems to be implicated in the prevention of carcinogenesis (Sawa, Nakao, Akaike, Ono, & Maeda, 1999) and atherosclerosis (Laranjinha, Almeida, & Madeira, 1994). Recently, a strong inhibitory effect of CGA on metalloproteinase (MMP-9) activity has also been shown, suggesting a role for this compound in cancer chemoprevention (Jin et al., 2005).

Regardless of nitrogen supply, 3,5-DCQA was the most abundant phenolic acid found in each organ of yarrow plants (Table 3). Compared to control plants, 3,5-DCQA increased by about 3- and 35-fold in leaf and root samples of nitrogen-starved plants, respectively. Similarly, 4,5-DCQA content in leaves and roots was significantly enhanced by nitrogen starvation, especially in root samples (Table 3). The amount of both DCQAs measured in leaves and inflorescences of control plants were comparable to those reported by Benedek et al. (2007). DCQAs are of the utmost interest from a medical viewpoint since they have been associated with the inhibition of HIV integrase (Zhu, Cordeiro, Atienza, Robinson, & Chow, 1999) and with the choleretic effect of yarrow extracts (Benedek et al., 2006). The dicaffeoylquinic acids are not as common in plant tissue as is CGA, and very little is known about their biosynthetic pathway (Mahesh et al., 2006). Other than in yarrow, 3,5-DCQA has been found in interesting amounts in coffee beans (Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004), in different organs of edelweiss (Schwaiger et al., 2006) and in flowering heads of *Arnica montana* cv. ARBO (Spitaler et al., 2006). In the latter study, the level of DCQA was positively correlated with altitude and a role in UV-B plant protection has been proposed.

Changes in phenolic acids content were associated with modification of PAL activity, the entry point enzyme into the phenylpropanoids pathway. A remarkable enhancement of PAL activity in leaf and root samples of nitrogen-starved compared to control plants was recorded (Fig. 4). Conversely, nitrogen depletion led to decreased PAL activity in inflorescence samples compared to the control treatment. A highly significant relationship was found between PAL activity and CGA content ($r = 0.72$, $p < 0.001$), suggesting that

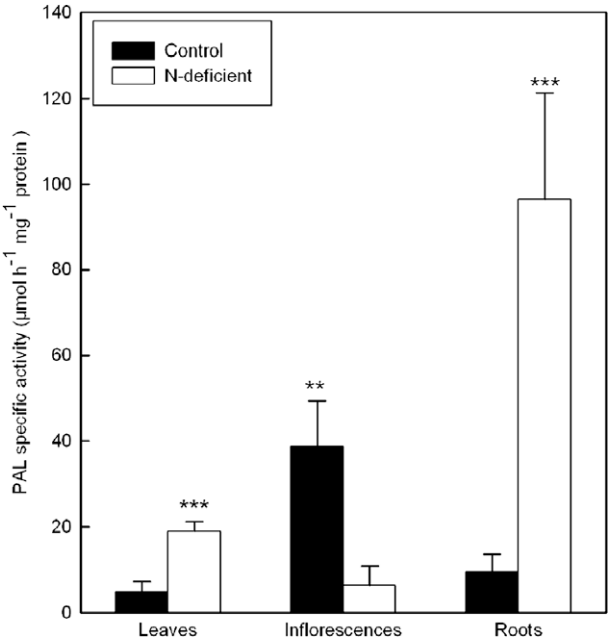

Fig. 4. PAL specific activity in different organs of control and nitrogen-starved (N-deficient) yarrow plants grown in hydroponics for 4 months. Each value is the mean ± SD ($n = 5$). ** and *** indicate significant differences according to the Student's *t*-test at the $p < 0.01$ and $p < 0.001$ levels, respectively.

increased PAL activity was mainly involved in CGA biosynthesis and accumulation. In accordance with our data, Shadle et al. (2003) reported that tobacco plants over-expressing PAL produce high levels of CGA. The ammonia released by the PAL reaction can be efficiently recycled through the GS/GOGAT system (Razal, Ellis, Singh, Lewis, & Towers, 1996) and the resulting nitrogen-free carbon skeletons can be directed into different phenolic biosynthetic pathways (e.g. Kováčik et al., 2007). In addition, it is known that stress conditions induce ROS formation and that phenolic compounds have strong antioxidant properties, in particular CGA (Grace & Logan, 2000). Based on these facts, we suggest that increased PAL activity, leading to CGA accumulation in yarrow plants grown under nitrogen deficient conditions, had the combined advantages of effective nitrogen recycling necessary to sustain (to some extent) plant growth and an increased protection against oxidative damage provided by CGA.

4. Conclusions

Knowing that caffeic acid derivatives (CGA and DCQAs) are important antioxidants for human health, the development of strategies to increase the content of these molecules in food and medicinal plants is required. Other than by engineering plants with constitutively high metabolite level, for instance by overexpressing enzymes of a specific biosynthetic pathway (Niggeweg et al., 2004; Canter et al., 2005), growing plants in hydroponics, with appropriate manipulation of nutritional and environmental factors involved in CGA and DCQAs biosynthesis and accumulation, may be an alternative way to maximise the yields of these compounds. Our results show that nitrogen starvation significantly increases phenylpropanoid metabolism in yarrow plants and the antioxidant capacity of the extracts, suggesting that hydroponics might be a suitable system for large scale production of caffeic acid derivatives from yarrow.

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