

Article

Nitrogen Starvation and Nitrate or Ammonium Availability Differently Affect Phenolic Composition in Green and Purple Basil

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Abstract: Basil (*Ocimum basilicum* L.) comprises green and purple cultivars, worldwide cultivated and appreciated for high contents of rosmarinic acid and anthocyanins, respectively. Although nitrogen (N) fertilization is needed for high yields, in basil it could have detrimental effects on the accumulation of phenolic compounds. In this study, plants of the cultivars ‘Italiano Classico’ (green) and ‘Red Rubin’ (purple) were grown in hydroponics and subjected to different nutritional treatments, consisting in N starvation, and nitrate (NO_3^-) or ammonium (NH_4^+) nutrition. Plant growth and nutritional status, estimated by the contents of NO_3^- , NH_4^+ , and amino acids in roots and leaves, were evaluated and put in relation with quality traits of basil leaves, such as chlorophyll content, antioxidant capacity, total phenols, the activity of phenylalanine ammonia-lyase, and the concentrations of individual (poly)phenolic acids and flavonoids. This study reveals that N starvation, as well as the availability of the two inorganic N forms, differently affect the phenolic composition in the two cultivars. Compared to plants grown in NO_3^- availability, in NH_4^+ availability, green basil showed a higher content of (poly)phenolic acids, while in purple basil, an increase in the contents of anthocyanins was detected. Overall, the study suggests that the management of NH_4^+ supply could contribute to enhance crop quality in hydroponics, and provides new knowledge about the relationship between N nutrition and phenolic metabolism in basil.

Keywords: basil; plant mineral nutrition; secondary metabolism; soilless agricultural system

1. Introduction

Basil (*Ocimum basilicum* L., *Lamiaceae*) is one of the most popular herbaceous crops, widely cultivated for culinary, industrial, and medicinal purposes [1]. Basil is considered an important natural source of secondary metabolites, including terpenes, phenylpropanoids, and phenolic compounds [2]. This species comprises a large number of cultivars, differing in morphological traits and in organoleptic and aromatic profiles. Very appreciated are the cultivars typical of the Mediterranean area, with large bright green leaves, as well as the purple varieties, characterized by the accumulation of anthocyanins in leaves and flowers [1,2].

Overall, basil contains a complex mixture of flavonoids and (poly)phenolic acids with antioxidant properties, among which rosmarinic and chicoric acids are the most abundant [3–5]. These compounds contribute to the technological quality of basil [3], and to some of the beneficial properties of leaf extracts, such as the cardioprotective and hypoglycemic activities [6]. Moreover, purple basil is also a very rich natural source of anthocyanins [7], plant polyphenolic pigments that improve the nutraceutical value of fruits and vegetables [8].

The metabolism of phenolic compounds, that participates in several physiological processes of the plant and in the plant-environment interactions, may be affected by agricultural practices [9,10]. To maximize yield, quality and phenol contents in basil crops, novel methodologies have been proposed, such as chemical treatments [11,12], UV-B exposure [13], and soilless systems [14,15]. Hydroponic cultivation offers several advantages for leafy crops, including all-year productivity, high yield per unit ground area, and low contamination from soil particles, pollutants, and pests [16]. In addition, this technique contributes to improve the antioxidant and nutraceutical properties of basil [17]. Moreover, the capability to strictly control the composition of the hydroponic solution appears very useful, especially as far as it concerns nitrogen (N) and potassium nutrients that significantly affect phenolic metabolism in this crop [18–20].

Nitrogen is an essential nutrient throughout plant growth, being a constituent of most of the main (macro)molecules required by plant metabolism, such as proteins, nucleic acids, ATP, NAD(P)H, chlorophyll, pigments, secondary metabolites, and hormones [21]. In plants, N deficiency induces inhibition of photosynthesis, stunted growth, chlorophyll loss, and leaf senescence. These responses are associated with an increase in protein degradation, a decline in the rate of the Calvin cycle, and, finally, with a rise in reactive oxygen species (ROS) that triggers oxidative stress [22]. Interestingly, N deficiency stimulates the phenylpropanoid metabolism, and, in particular, induces the accumulation of phenylalanine ammonia-lyase (PAL, EC: 4.3.1.24), the first enzyme of the pathway [23,24]. This response suggests that, during N deficiency, the decrease in the metabolic requirements for plant growth promotes the accumulation of C-based secondary metabolites [18]. Moreover, it has been proposed that PAL activity, by releasing NH_4^+ and cinnamic acid from phenylalanine, increases in plants the contents of N and precursors for phenylpropanoid biosynthesis [25]. In addition, it has been suggested that the accumulation of phenolic compounds could also participate in the protection against oxidative stress [19,22].

Nitrate (NO_3^-) is the most abundant mineral form of N in agricultural soils, mainly due to the processes of mineralization, i.e., the conversion of organic forms of N to ammonium (NH_4^+), and of nitrification, i.e., the oxidation of NH_4^+ to NO_3^- , both conducted by soil micro-organisms [26]. In hydroponics, these processes are slowed down due to a lower presence of microorganisms in the nutrient solution, also guaranteed by frequent renewal and, therefore, it is possible to provide plants a high availability of NH_4^+ . This strategy could reduce the leaching of NO_3^- into the environment as well as its accumulation in the edible parts of leafy crops [19]. However, the $\text{NO}_3^-/\text{NH}_4^+$ ratio in fertilizer inputs affects plant growth, biomass allocation, and the responses to abiotic stresses [21]. Ammonium can be assimilated more promptly than NO_3^- , but when provided as sole N form, excessive amounts of this cation can result in detrimental effects for plant growth. Although the complexity of NH_4^+ toxicity in plants is not yet fully elucidated, it surely involves ionic imbalances, interference with photosynthesis, and oxidative stress [27]. Hence, paradoxically, the stresses imposed by N deficiency and by excess of NH_4^+ share common traits.

Considering the high antioxidant properties of the main basil phenolic compounds [3], it is possible that their metabolism contributes to the plant responses to nutritional imbalances that lead to oxidative stress. This hypothesis is also supported by the observation that, in purple basil, the photoprotection conferred by foliar anthocyanins mitigates the effects of boron toxicity [28]. Interestingly, in a study conducted in green and red lettuce (*Lactuca sativa* L.) it was observed that low N availability increases the concentrations of phenolic compounds, with a relative increase more pronounced in the red cultivar, supporting a photoprotective role of anthocyanins in leafy crops during nutritional stresses [29]. On these bases, it is conceivable that in green and purple basil the phenolic metabolism could be differently affected by the forms of mineral N, but, to our best knowledge, these responses have only partially been described.

In basil crops, N fertilization is required to sustain high productivity [30]; nevertheless, high N inputs can induce a decrease in the levels of rosmarinic acid [18,19,31]. Moreover, in plants of green basil at bloom stage, previously exposed to increasing $\text{NH}_4^+/\text{NO}_3^-$ ratios, a decrease in biomass and in

rosmarinic acid accumulation was observed [19]. In addition, in vitro experiments recently showed that the $\text{NH}_4^+/\text{NO}_3^-$ ratios differently affect green and purple basil, inducing distinct changes in lipid peroxidation, PAL activity, total phenol contents, and antioxidant activities [31]. However, a systematic description of how the type of mineral N form affects plant physiology and phenolic composition is still lacking.

The aim of the present work was the study of green and purple basil, grown in hydroponics, and exposed to three nutritional treatments, consisting, respectively, in N starvation or supply of either NO_3^- or NH_4^+ . This comparison was conducted by assessing a few physiological responses, such as biomass allocation and plant nutritional status, as well as the changes in a few biochemical parameters related to leaf quality, such as the chlorophyll content, the antioxidant capacity, and the total phenol contents. Finally, the activity of PAL as well as the phenolic composition of leaves were evaluated. Overall, the results highlighted different metabolic responses in green and purple basil under the different N nutritional treatments, and provide, for the first time, information about the changes induced in the contents of individual (poly)phenolic acids and flavonoids.

2. Materials and Methods

2.1. Plant Material and Nutritional Treatments

Young plants of basil (*Ocimum basilicum* L.) of the cultivars 'Italiano Classico' (IC, green basil) and 'Red Rubin' (RR, purple basil) grown in soil were obtained from the commercial nursery Agri Brianza Garden Store (Concorezzo, MB, Italy). For each cultivar, plants at uniform growth phase, corresponding to the full development of the 2nd leaf, were selected and, in order to remove soil contaminants, their root systems were thoroughly washed with running distilled water. The plants were transferred into a hydroponic system where six plants were anchored onto a floating polystyrene holder in a 5-L tank. The hydroponic culture was conducted in a growth chamber equipped with a ventilation system, imposing a day/night regime of 16/8 h, photosynthetic photon flux density of ca. $450 \mu\text{mol m}^{-2} \text{s}^{-1}$, using two PhytoLED GX400 Full spectrum (265 W, Phytolite—PHT Trading sa, Corteglia, Switzerland), each composed by eight trays equipped with seven white (broad nm range, 6500 K), two blue (470 nm), and three red (630 nm) LEDs (Light Emitting Diode), with uniform distribution (LED expected lifespan of 50.000 h), 26/22 °C and relative humidity (RH) of 60/55%. The protocol for hydroponic cultivation is outlined in Figure 1a. In detail, plants were treated for 2 d with 4 mM CaSO_4 to facilitate root adaptation to the hydroponic condition. To allow the reaching of the adult vegetative phase (Figure 1b), plants were grown in low N nutrition for 7 d by transferring in the control solution (2.24 mM K_2SO_4 , 1 mM MgSO_4 , 0.52 mM KH_2PO_4 , 0.4 mM CaSO_4 , 60 μM Fe-EDTA, 40 μM H_3BO_3 , 25 μM KCl, 3.2 μM MnSO_4 , 0.8 μM CuSO_4 , 0.8 μM Na_2MoO_4 , 0.8 μM ZnSO_4) added with 0.25 mM $\text{Ca}(\text{NO}_3)_2$ and 62.5 μM $(\text{NH}_4)_2\text{SO}_4$. Plants were then grown for additional 3 d in the control solution without N nutrient (N starvation) to foster the consumption of N plant reserves. Finally, plants were exposed to three different N treatments for 5 d, which consisted in the control solution: (1) without N addition—control, [con]; (2) added with 5 mM $\text{Ca}(\text{NO}_3)_2$ —total 10 mM NO_3^- , [nit]; (3) added with 5 mM $(\text{NH}_4)_2\text{SO}_4$ —total 10 mM NH_4^+ , [amm]. The pH of all solutions was adjusted to 6.1. All the hydroponic solutions were continuously aerated by an electric pump and changed every two or three days. Before sampling, the plant root systems were rapidly washed in distilled water and rinsed twice in aerated ice-cold solution (10 mM K_2SO_4 , 0.4 mM CaSO_4 , 1 mM MES-BTP pH 6.1) in order to remove from the apoplasmic space the adsorbed ions. At sampling, roots were rinsed again with distilled water and gently blotted with paper towels. Roots and leaves were collected and weighed separately (Supplementary Table S1). Each biological sample was composed of roots or leaves collected from four plants. Samples were immediately frozen and ground (mortar and pestle) in liquid N_2 to obtain a fine uniform powder, which was stored at -80°C . Aliquots of each sample were used for the following analyses.

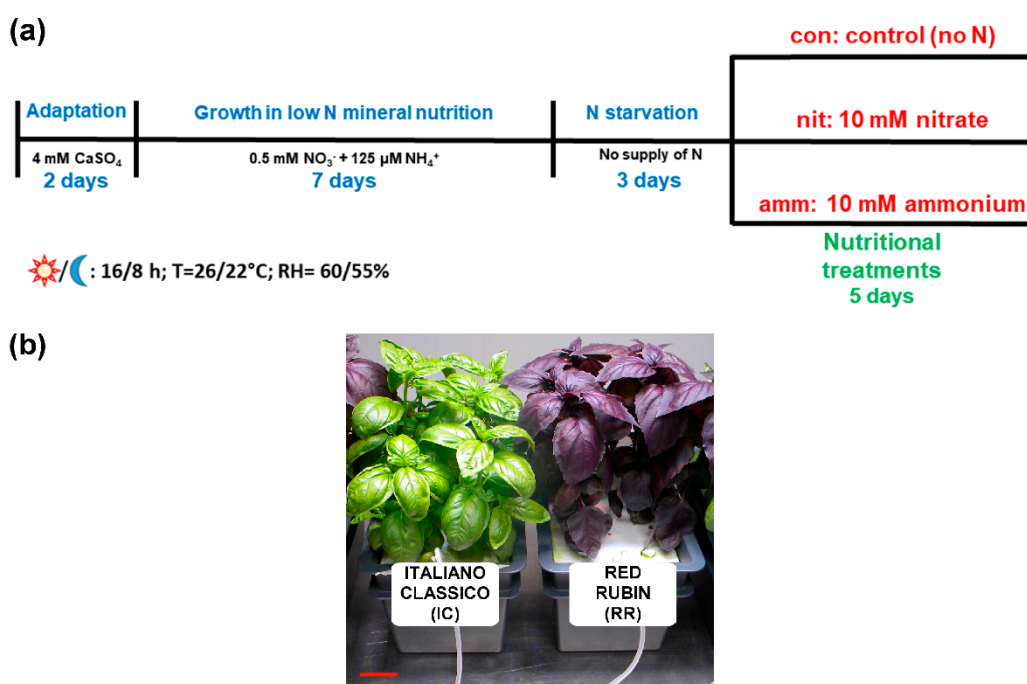


Figure 1. Hydroponics of green and purple basil. (a) Outline of the experimental hydroponic design. Young basil plants grown in soil were adapted (2 d) to the hydroponic condition and then grown for 7 d in low N mineral nutrition until the adult vegetative phase was reached. After 3 d of N starvation, plants were exposed for 5 d to: absence of N—control [con], 10 mM NO₃⁻—nitrate [nit], or 10 mM NH₄⁺—ammonium [amm]. For details, see the Materials and Methods section. (b) Basil plants of the green ‘Italiano Classico’ (IC, on the left) and of the purple ‘Red Rubin’ (RR, on the right) cultivars grown in hydroponics up to the adult vegetative phase; red scale bar = 5 cm.

2.2. Determination of the Contents of Nitrate, Ammonium, and Total Amino Acids in Roots and Leaves

Nitrate was extracted from root or leaf samples as previously described [24], with some modifications. Briefly, the samples were boiled at 100 °C for 20 min in four volumes (*v/w*) of distilled water and centrifuged twice at 5000× *g* for 15 min. An aliquot of the obtained supernatants was used for the determination of NO₃⁻ concentration, according to Cataldo et al. [32].

The NH₄⁺ contents in roots and leaves were measured as previously described [33]. Briefly, samples were homogenized in five volumes (*v/w*) of ice-cold 10 mM formic acid (FA), shaken for 15 min at 4 °C, and centrifuged at 14,000× *g* for 10 min at 4 °C. Supernatants were filtered (0.45 μm Millex HV, Merck Life Science, Milan, Italy), and an aliquot was used for the determination of the NH₄⁺ concentration, according to the colorimetric method (*o*-phthalaldehyde reaction) proposed by Coskun et al. [34].

Amino acids were extracted from leaf and root samples as previously described [24], with some modifications. Briefly, samples were homogenized in eight volumes (*v/w*) of ice-cold 0.5 M perchloric acid (PCA), shaken for 15 min at 4 °C, and centrifuged twice at 13,000× *g* for 15 min at 4 °C. Finally, the supernatants were added with KOH to reach pH 7.6 and centrifuged to remove excess KClO₄. Amino acid concentrations were measured by the ninhydrin method [35]. The values were determined referring to a calibration curve of leucine (Leu) and expressed as μmol Leu g⁻¹ FW.

All of the analyses were replicated on three independent biological samples (*n* = 3).

2.3. Determination of the Contents of Chlorophyll, Total Phenols, and Antioxidant Capacity in Leaves

Chlorophyll was extracted from leaves by homogenizing the frozen powder samples in 10 volumes (*v/w*) of pre-cooled (−20 °C) 80% (*v/v*) acetone. Samples were shaken in the dark at 4 °C for 25 min and centrifuged at 4000× *g* for 10 min at 4 °C. Pellets were then washed in three volumes of 80% (*v/v*)

acetone ($-20\text{ }^{\circ}\text{C}$) and centrifuged again in the same conditions as above. After combining the two supernatants, aliquots were diluted 1:10 (v/v) and used to determine the chlorophyll concentrations by the colorimetric method described by Arnon [36].

The contents of total phenols and antioxidant capacity in leaves were evaluated as previously described [5], with some modifications. Briefly, samples were homogenized in 1.5 volumes (v/w) of methanol and centrifuged at $10,000\times g$ for 30 min. Pellets were then suspended in 0.5 volumes (v/w) of 70% (v/v) methanol and centrifuged at $10,000\times g$ for 30 min. After combining the two supernatants, aliquots were diluted 1:5 (v/v) and used to determine the concentration of total phenols by the microscale Folin–Ciocalteu procedure [37]. The absorbance at 765 nm was referred to a calibration curve of gallic acid (GA) and expressed as mg GA equivalents (GAE) g^{-1} FW. The antioxidant capacity was determined in the same extracts according to the colorimetric method described by Prieto et al. [38]. The absorbance at 695 nm was referred to a calibration curve of ascorbic acid (AA) and expressed as μmol AA equivalents (AAE) g^{-1} FW.

All of the analyses were replicated on three independent biological samples ($n = 3$).

2.4. Determination of *In Vitro* Total PAL Activity in Leaves

The *in vitro* assays of PAL activity were conducted as previously described [39]. Briefly, aliquots of the frozen powdered leaf samples were homogenized in four volumes (v/w) of extraction buffer [100 mM Tris-HCl, 2 mM $\text{Na}_2\text{-EDTA}$, 5 mM ascorbic acid, 1 mM PMSF, 5 mM 2-mercaptoethanol, 10% (w/w) poly(vinylpyrrolidone), pH 8.8], filtered through cheesecloth, and centrifuged at $15,000\times g$ for 30 min at $4\text{ }^{\circ}\text{C}$. The *in vitro* assay was conducted by adding to a mix buffer (1 mL total volume; 100 mM Tris-HCl, pH 8.8, plus 20 mM phenylalanine; $38\text{ }^{\circ}\text{C}$) an aliquot of the obtained supernatants; the reaction was stopped, after 0, 30, or 60 min, by addition of 250 μL of 6 N HCl. After centrifugation, one unit of PAL activity was expressed as the amount of enzyme causing an increase of 0.01 in absorbance at 290 nm of the supernatant, corresponding to 3.09 nmol of cinnamic acid (CA) h^{-1} . PAL specific activity was expressed on the basis of the total protein content, determined by the Bradford assay [40] (Micro-Bio-Rad Protein Assay; Bio-Rad Laboratories, Segrate, Italy).

The analysis was replicated on three independent biological samples ($n = 3$).

2.5. Determination of the Contents of Individual (Poly)phenolic Acids and Flavonoids in Leaves

The analysis of the contents of individual (poly)phenolic acids and flavonoids in leaves was conducted in the same extract. Briefly, phenolic compounds were purified by extraction in $\text{CHCl}_3\text{:CH}_3\text{OH:H}_2\text{O}$ (12:5:1 $v/v/v$, pH 2 by addition of FA) and purification by phase partitioning, according to Tattini et al. [41]. Samples were then filtered by Millex HV (0.45 μm , Merck Life Science, Milan, Italy), diluted, and analyzed by LC-ESI-MS/MS (Liquid Chromatography – ElectroSpray Ionization – tandem mass spectrometry) as previously described [5]. Briefly, samples were analyzed by HPLC (1200 series; Agilent Technologies Italia, Cernusco sul Naviglio, Italy) coupled with ESI-Q-TOF (ESI-Quadrupole-Time Of Flight mass spectrometer, 6520, Agilent Technologies) on a XDB-C18 column (2.1 \times 50 mm, 1.8 μm) in acidic condition [0.1% (v/v) FA] at 200 $\mu\text{L min}^{-1}$, by discontinuous acidified acetonitrile [0.1% (v/v) FA] gradient [0–2 min at 5%, 2–15 min to 15%, 15–45 min to 45%]. The MS analysis of (poly)phenolic acids and flavonoids was conducted in negative (-3000 V , $350\text{ }^{\circ}\text{C}$) and positive ($+3500\text{ V}$, $350\text{ }^{\circ}\text{C}$) mode, respectively. The targeted MS/MS analyses were performed setting collision energy at 15 V and 25 V in negative and positive mode, respectively (see Supplementary Table S2 for further details). The quantification of compounds was done using EIC (extracted ion current, $\pm 0.05\text{ Da}$) from MS spectra, and referring to an external calibration curve. In detail, caffeic acid was used to calibrate salvianic acid A, rosmarinic acid was used to calibrate itself, chicoric acid was used to calibrate itself and salvianolic acid K, salvianolic acid B was used to calibrate salvianolic acid L (isomer), cyanidin rutinoside was used as standard for flavonoids. Quantitative analyses were conducted on three biological replicates ($n = 3$), verifying molecule assignments by MS/MS analysis in each condition.

2.6. Statistical Analysis

Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA). Differences in plant biomass and in the leaf to root ratio (L/R) were evaluated by Student's t-test within each nutritional treatment ($p < 0.05$). For all other data, the normality and the equal variance were checked using the Shapiro–Wilk test and the Levene Median test, respectively. Data were then compared using ANOVA, with Tukey's multiple comparison test ($p < 0.05$). In order to assess the influence of the nutritional treatments within one cultivar, one-way ANOVA was applied. In order to assess the effects of individual factors (nutritional treatments, cultivars) and their interaction, two-way ANOVA was applied. Where the interaction between the two factors ($A \times B$) was significant ($p < 0.05$), all conditions were subjected to one-way ANOVA, comparing all of them to each other. On the contrary, where $A \times B$ interaction was not significant, the effect of the nutritional treatments and of the cultivars was evaluated separately. Data in the tables and figures were calculated as arithmetic means with standard error of the means (SEM).

3. Results

3.1. Experimental Hydroponic Design and N Nutritional Treatments

Young basil plants of the green cultivar 'Italiano Classico' (IC) and of the purple one 'Red Rubin' (RR) were grown in the experimental hydroponic system outlined in Figure 1a. Starting from soil-grown young plants, the protocol foresaw a first phase (2 d) of root adaptation to hydroponics, followed by 7 d in low N condition. This time period was adopted in order to obtain adult plants in a vegetative phase (Figure 1b) close to the time of harvest for basil crops in the Mediterranean area [2], and to reduce the possible residual effects of the previous cultivation in soil. These plants were used to study the biochemical and physiological responses evoked by the different N availability. After a short (3 d) period of N starvation to reduce endogenous N reserves, plants were exposed for 5 d to three nutritional treatments: absence of N—control [con]; 10 mM NO_3^- —nitrate [nit]; 10 mM NH_4^+ —ammonium [amm].

3.2. Effects of Mineral N Forms on Plant Growth and Nutritional Status of Green and Purple Basil

Plant growth was evaluated as fresh biomass of roots and leaves; biomass allocation was estimated as ratio of Leaf fresh weight/Root fresh weight (L/R, Figure 2). Green basil (IC) was characterized by higher biomass, in both roots and leaves, than the purple cultivar (RR) (Figure 2a–d; Supplementary Table S1). However, the N nutritional treatments had similar effects on plant growth in the two cultivars. Overall, the availability of NO_3^- [nit] sustained the highest biomass accumulation, but with different effects in roots and leaves compared to the two other N nutritional treatments. In comparison to N starvation [con], NO_3^- nutrition [nit] elicited positive effects only in leaves, where the biomass was by 46% ($p < 0.001$) and by 23% ($p = 0.017$) higher in IC and RR, respectively (Figure 2c,d). When compared to NH_4^+ availability [amm], NO_3^- availability [nit] sustained higher biomass accumulation in both organs. This effect was more pronounced in roots (by +49% and by +31% in IC and RR, $p = 0.015$ and $p = 0.033$, respectively; Figure 2a,b), than in leaves (+33% and +19%, $p = 0.004$ and $p = 0.041$, respectively; Figure 2c,d). Exposure of plants to NH_4^+ [amm] did not seem to induce changes in biomass accumulation compared to N starvation [con] in any cultivar. However, concerning L/R, NH_4^+ nutrition [amm] induced an increase compared to N starvation [con] in biomass allocation in both IC (+26%, $p = 0.010$) and RR (+14%, $p = 0.033$), mainly attributable to lesser root growth (Figure 2e,f).

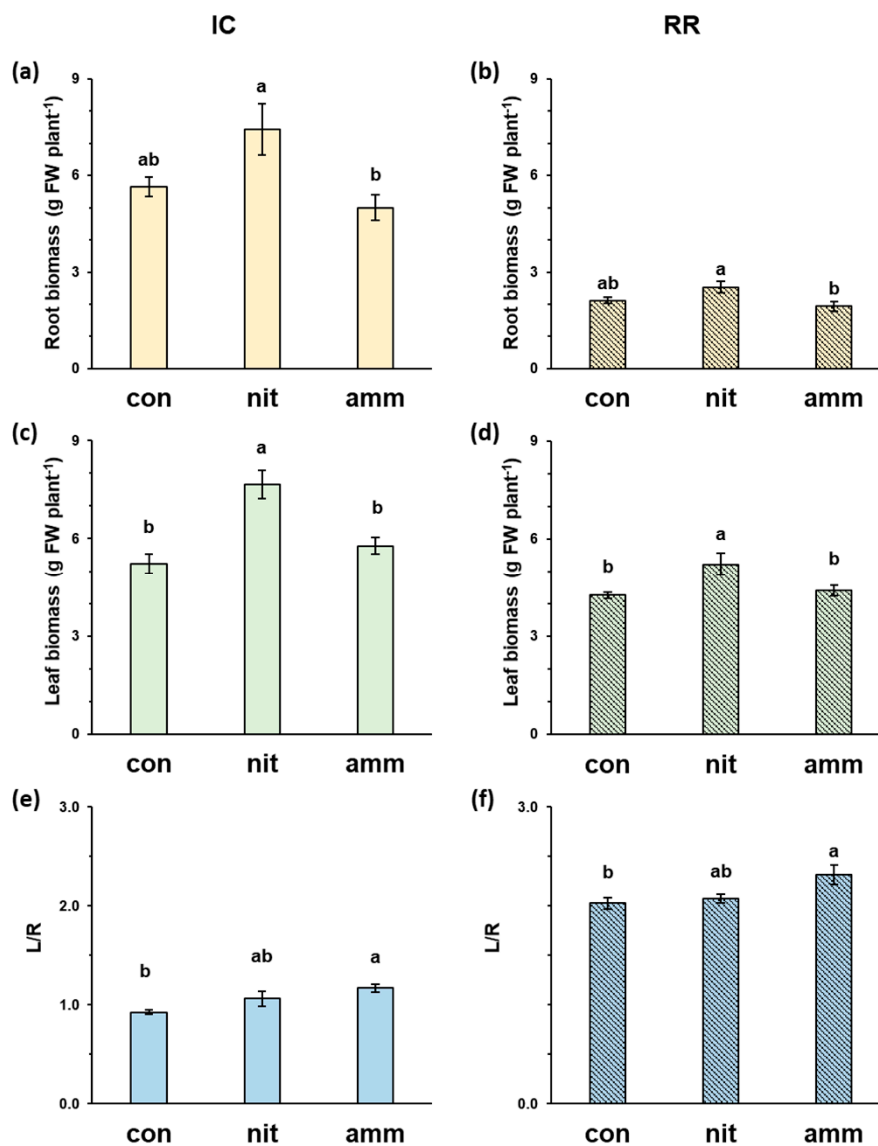


Figure 2. Growth of green and purple basil cultivars exposed to different N nutritional treatments. Plant growth was evaluated as fresh weight (FW) of roots [(a,b), yellow bars] and leaves [(c,d), green bars] per plant (g FW plant⁻¹) and as Leaf/Root fresh weight ratio [(L/R), (e,f), blue bars] in the green 'Italiano Classico' [IC, (a,c,e), open bars] and in the purple 'Red Rubin' [RR, (b,d,f), hatched bars] cultivars. Plants were exposed for 5 d to absence of N—control [con], 10 mM NO₃⁻—nitrate [nit], or 10 mM NH₄⁺—ammonium [amm]. Values are the means ± SEM (n = 6). Statistical significance within each cultivar was assessed by one-way ANOVA and Tukey's test. Various letters indicate significant differences among the different nutritional treatments (*p* < 0.05).

Taken together, the results highlight that the different N nutritional treatments had significant effects on basil growth, similar in green and purple cultivars.

In order to evaluate whether the differences in plant growth were related to changes in the nutritional and metabolic status of the plants, the contents of NO₃⁻, NH₄⁺, and total amino acids were measured in roots and leaves of IC and RR plants exposed to the three experimental N nutritional treatments (Table 1).

Table 1. Evaluation of plant N nutritional status by determination of the contents of NO_3^- , NH_4^+ , and total amino acids in roots (a) and leaves (b). Basil plants of the green ‘Italiano Classico’ (IC) and of the purple ‘Red Rubin’ (RR) cultivars were exposed for 5 d to absence of N—control [con], 10 mM NO_3^- —nitrate [nit], or 10 mM NH_4^+ —ammonium [amm]. CV: cultivar. AA: total amino acids ($\mu\text{mol Leu g}^{-1}$ FW). Values are means \pm SEM (standard error of the mean, $n = 3$). Statistical significance was assessed by two-way ANOVA and Tukey’s test. Where interaction (N treatment \times cultivar, A \times B) was significant (** = $p < 0.001$; * = $p < 0.05$), data were subjected to one-way ANOVA (lowercase letters). Where interaction was not significant (n.s.) the effect of the N treatments (italic letters) and cultivars (uppercase letters) was evaluated separately. Various letters indicate significant differences among the different nutritional treatments ($p < 0.05$).

(a) Roots						
	CV	con mean \pm SEM	nit mean \pm SEM	amm mean \pm SEM	Two-Way ANOVA	F statistic
NO_3^- ($\mu\text{mol g}^{-1}$ FW)	IC	4.59 \pm 0.14 c	42.28 \pm 0.38 a	4.23 \pm 0.17 cd	N treatment (A)	8128.43 **
	RR	2.87 \pm 0.13 d	32.93 \pm 0.53 b	3.46 \pm 0.28 cd	Cultivar (B)	249.29 **
					A \times B	118.14 **
NH_4^+ ($\mu\text{mol g}^{-1}$ FW)	IC	2.55 \pm 0.26 bc	0.59 \pm 0.11 d	4.76 \pm 0.29 a	N treatment (A)	115.09 **
	RR	1.80 \pm 0.26 c	0.72 \pm 0.04 d	3.13 \pm 0.22 b	Cultivar (B)	17.89 *
					A \times B	8.12 *
AA ($\mu\text{mol g}^{-1}$ FW)	IC	1.76 \pm 0.02 b	2.21 \pm 0.18 b	16.71 \pm 2.17 a	N treatment (A)	102.75 **
	RR	1.63 \pm 0.07 b	1.70 \pm 0.11 b	16.20 \pm 1.90 a	Cultivar (B)	0.16 n.s.
					A \times B	0.02 n.s.
(b) Leaves						
	CV	con mean \pm SEM	nit mean \pm SEM	amm mean \pm SEM	Two-Way ANOVA	F statistic
NO_3^- ($\mu\text{mol g}^{-1}$ FW)	IC	0.80 \pm 0.80 b	46.84 \pm 0.82 a	0.78 \pm 0.39 b	N treatment (A)	252.36 **
	RR	5.26 \pm 1.81 b	40.04 \pm 3.88 a	7.77 \pm 2.30 b	Cultivar (B)	0.86 n.s.
					A \times B	6.44 *
NH_4^+ ($\mu\text{mol g}^{-1}$ FW)	IC	3.13 \pm 0.44 a B	2.18 \pm 0.07 b B	5.01 \pm 0.08 a B	N treatment (A)	14.21 **
	RR	4.59 \pm 0.18 a A	3.43 \pm 0.38 b A	4.59 \pm 0.69 a A	Cultivar (B)	6.21 *
					A \times B	3.75 n.s.
AA ($\mu\text{mol g}^{-1}$ FW)	IC	6.93 \pm 0.57 c	6.85 \pm 1.17 c	44.99 \pm 2.18 a	N treatment (A)	404.27 **
	RR	6.76 \pm 0.27 c	6.82 \pm 0.59 c	37.14 \pm 2.17 b	Cultivar (B)	5.58 *
					A \times B	5.19 *

The contents of NO_3^- were similarly affected in roots and leaves, also showing similarities in the two cultivars (Table 1). Plants exposed to N starvation [con] or NH_4^+ nutrition [amm] were characterized by low and comparable levels of the anion, whereas plants exposed to nitric nutrition [nit] showed a high increase in the levels of NO_3^- in both roots (+821% and +1047% compared to the controls in IC and RR, respectively) and leaves (+5755% and +661% compared to the controls in IC and RR, respectively).

The contents of NH_4^+ showed distinct trends in roots and leaves, with differences between the two cultivars (Table 1). In roots, the NH_4^+ levels were affected by the treatments, being lowest in the NO_3^- -treated plants [nit], intermediate in the N-starved plants [con], and highest in plants exposed to NH_4^+ nutrition [amm] (+87% and +74% compared to the controls in IC and RR, respectively). Moreover, in NH_4^+ nutrition [amm] the root content of the cation was higher in IC than in RR (+52%). In leaves, the main difference concerned the decrease of NH_4^+ contents in [nit] plants, and the higher contents of NH_4^+ in the purple cultivar. In order to better understand the specific behavior of the two cultivars, a multiple comparison of the leaf NH_4^+ contents among N treatments within each cultivar was performed. In RR, the leaf NH_4^+ content was not affected by the N nutritional treatments. Differently, in IC leaves the NH_4^+ content in [amm] plants increased and was higher compared to both [con] plants (+60%, $p = 0.005$) and [nit] plants (+130%, $p < 0.001$).

Finally, the NH_4^+ nutrition [amm] induced a strong accumulation of amino acids in the two cultivars in both roots (+849% and +893% compared to the controls in IC and RR, respectively) and leaves (+549% and +449% compared to the controls in IC and RR, respectively). In particular, in NH_4^+ -treated plants [amm] the content of amino acids in the leaves was higher (+21%) in IC than in RR (Table 1).

Overall, the results show that the different N nutritional treatments had different effects on the plant nutritional status and metabolic adaptations in the green and purple basil cultivars considered.

3.3. Effects of Mineral N Forms on Total Chlorophyll Content, Antioxidant Capacity, Total Phenols, and PAL Activity in Leaves of Green and Purple Basil

In the light of the changes observed in the plant nutritional status, the study analyzed, in IC and RR, the possible effects of the N nutritional treatments on parameters that affect leaf quality, such as chlorophyll content, the antioxidant capacity of methanolic extracts, and total phenols. In order to obtain more information about phenolic metabolism, PAL activity was also evaluated (Table 2).

The results show that the chlorophyll content was not significantly affected by the N availability and was higher in RR than in IC plants.

Conversely, the leaf antioxidant capacity was similarly affected in the two cultivars by the N nutritional treatments, resulting higher in [amm] than in [nit] plants (+19% and +28% in IC and RR, respectively).

Other parameters were affected by the N nutritional treatments and were different in the green or purple cultivars. In particular, in IC plants the contents of total phenols significantly decreased only in response to NO_3^- nutrition [nit] (−34% compared to the controls), whereas the availability of N, supplied both in the forms of either NO_3^- or NH_4^+ , induced a significant decrease in PAL activity, compared to the control plants [con] (−64% and −54% in NO_3^- and NH_4^+ nutrition, respectively). RR plants showed a divergent scenario. The total phenol contents were higher than in IC in all conditions, as well as PAL activity was higher in the presence of inorganic N forms. Interestingly, all these parameters decreased significantly in plants exposed to NO_3^- nutrition [nit] (−29% and −20% for TP and PAL, respectively), but remained unchanged in the NH_4^+ -fed plants [amm], and comparable to those observed in the control ones [con] (Table 2).

Table 2. Evaluation of total chlorophyll content (Chl), antioxidant capacity (AC), total phenols (TP), and PAL activity (PAL) in green and purple basil leaves. Plants of the green cultivar ‘Italiano Classico’ (IC) and of the purple one ‘Red Rubin’ (RR) were exposed for 5 d to absence of N—control [con], 10 mM NO₃[−]—nitrate [nit], or 10 mM NH₄⁺—ammonium [amm]. CV: cultivar. GAE: gallic acid equivalents; AAE: ascorbic acid equivalents, CA: cinnamic acid. Values are means ± SEM (standard error of the mean, n = 3). Statistical significance was assessed by two-way ANOVA and Tukey’s test. Where interaction (N treatment × cultivar, A × B) was significant (** = *p* < 0.001; * = *p* < 0.05), data were subjected to one-way ANOVA (lowercase letters). Where interaction was not significant (n.s.), the effect of the N treatments (italic letters) and cultivars (uppercase letters) was evaluated separately. Various letters indicate significant differences among the different nutritional treatments (*p* < 0.05).

Parameter	CV	Con Mean ± SEM	nit Mean ± SEM	amm Mean ± SEM	Two-Way ANOVA F Statistic	
Chl (mg g ^{−1} FW)	IC	1.07 ± 0.07 B	1.05 ± 0.04 B	1.05 ± 0.03 B	N treatment (A)	2.35 n.s.
	RR	1.20 ± 0.09 A	1.05 ± 0.03 A	1.28 ± 0.03 A	Cultivar (B)	7.10 *
					A × B	2.31 n.s.
AC (μmol AAE g ^{−1} FW)	IC	35.6 ± 2.8 <i>a</i>	23.9 ± 1.1 <i>c</i>	28.5 ± 2.0 <i>b</i>	N treatment (A)	21.30 **
	RR	33.3 ± 1.1 <i>a</i>	23.3 ± 1.1 <i>c</i>	29.8 ± 1.2 <i>b</i>	Cultivar (B)	0.17 n.s.
					A × B	0.60 n.s.
TP (mg GAE g ^{−1} FW)	IC	5.48 ± 0.55 <i>aB</i>	3.62 ± 0.22 <i>bB</i>	4.52 ± 0.08 <i>aB</i>	N treatment (A)	24.46 **
	RR	6.71 ± 0.05 <i>aA</i>	4.77 ± 0.25 <i>bA</i>	6.31 ± 0.19 <i>aA</i>	Cultivar (B)	38.05 **
					A × B	0.79 n.s.
PAL (nmol CA mg ^{−1} _{prot} h ^{−1})	IC	535 ± 16 <i>ab</i>	190 ± 9 <i>c</i>	245 ± 26 <i>c</i>	N treatment (A)	66.38 **
	RR	603 ± 10 <i>a</i>	485 ± 9 <i>b</i>	549 ± 24 <i>ab</i>	Cultivar (B)	180.09 **
					A × B	20.33 **

Overall, the results show that the availability of NO_3^- or NH_4^+ in hydroponic solutions had different effects on phenol metabolism in the leaves of green or purple basil cultivars. This observation opens the possibility that, in green and purple basil, the two inorganic N forms may elicit distinct modulation. These different responses may possibly exploit the peculiar physiological roles of the different classes of phenols, in order for the plant to better adapt to specific N nutritional treatments. To better investigate this hypothesis, the individual composition of (poly)phenolic acids and flavonoids, being among the most important phenols that affect basil quality, was evaluated.

3.4. Effects of Mineral N Forms on the Contents of Individual (Poly)phenolic Acids in Leaves of Green and Purple Basil

The evaluation of the contents of individual (poly)phenolic acids in leaves of green and purple basil exposed to the different N nutritional treatments was conducted by LC-ESI-MS/MS analyses. This approach allowed to quantify five compounds (Figure 3), whose molecular characterization is detailed in Supplementary Table S2.

In detail, salvianic acid A and salvianolic acid L were detected only in IC leaves (Figure 3a,b), while the other molecules were quantifiable in both green and purple basil. Among them, salvianolic acid K was present in low abundance (Figure 3c), while chicoric and rosmarinic acids (Figure 3d,e) were the most abundant, accounting for about more than 15% and 75% of the total, respectively, in both cultivars. The contents of total (poly)phenolic acids were calculated as the sum of the contents of the individual molecules (Figure 3f).

Interestingly, the contents of the individual (poly)phenolic acids were affected differently by the N treatments, also showing different trends in IC and RR leaves.

In green basil (IC), the contents of salvianic acid A did not change in relation to the N nutritional treatments, whereas salvianolic acid L decreased in response to N availability, supplied as both NO_3^- and NH_4^+ (-46% and -42% , $p = 0.002$ and $p = 0.003$, respectively) (Figure 3a,b). The leaf contents of salvianolic acid K and of chicoric and rosmarinic acids were significantly lower in plants exposed to NO_3^- nutrition [nit] than in the controls [con] (-48% , -46% , and -57% , $p = 0.039$, $p = 0.009$, and $p = 0.001$, respectively), but were unaltered in NH_4^+ -fed plants [amm] (Figure 3c,d,e). Considering the higher abundance of these last molecules, this response is well summarized by the values of total contents of (poly)phenolic acids (-53% in NO_3^- -fed compared to control plants, $p = 0.001$, Figure 3f).

This response was less broad in the purple basil cultivar. In RR leaves, salvianolic acid K dropped to undetectable levels upon plant exposure to N availability, whereas the contents of chicoric acid were unaffected by the N nutritional treatments (Figure 3c,d). Only rosmarinic acid showed a trend comparable to that observed in the green cultivar. The content of this compound significantly decreased in NO_3^- -fed plants [nit] compared to the control ones [con] (-60% , $p = 0.041$), but, however, in NH_4^+ -fed plants [amm], it reached an intermediate value between those observed in the control [con] and in the NO_3^- [nit] treatments (-33% and $+66\%$, respectively, not significant, Figure 3e). Once again, this trend was well mirrored by that of total (poly)phenolic acids (-56% in NO_3^- -fed plants compared to the control ones, $p = 0.030$, Figure 3f).

Taken together, the results show that the type of N inorganic form influenced in specific ways the accumulation of the individual (poly)phenolic acids in basil leaves, and induced different metabolic responses in the green and purple cultivars considered.

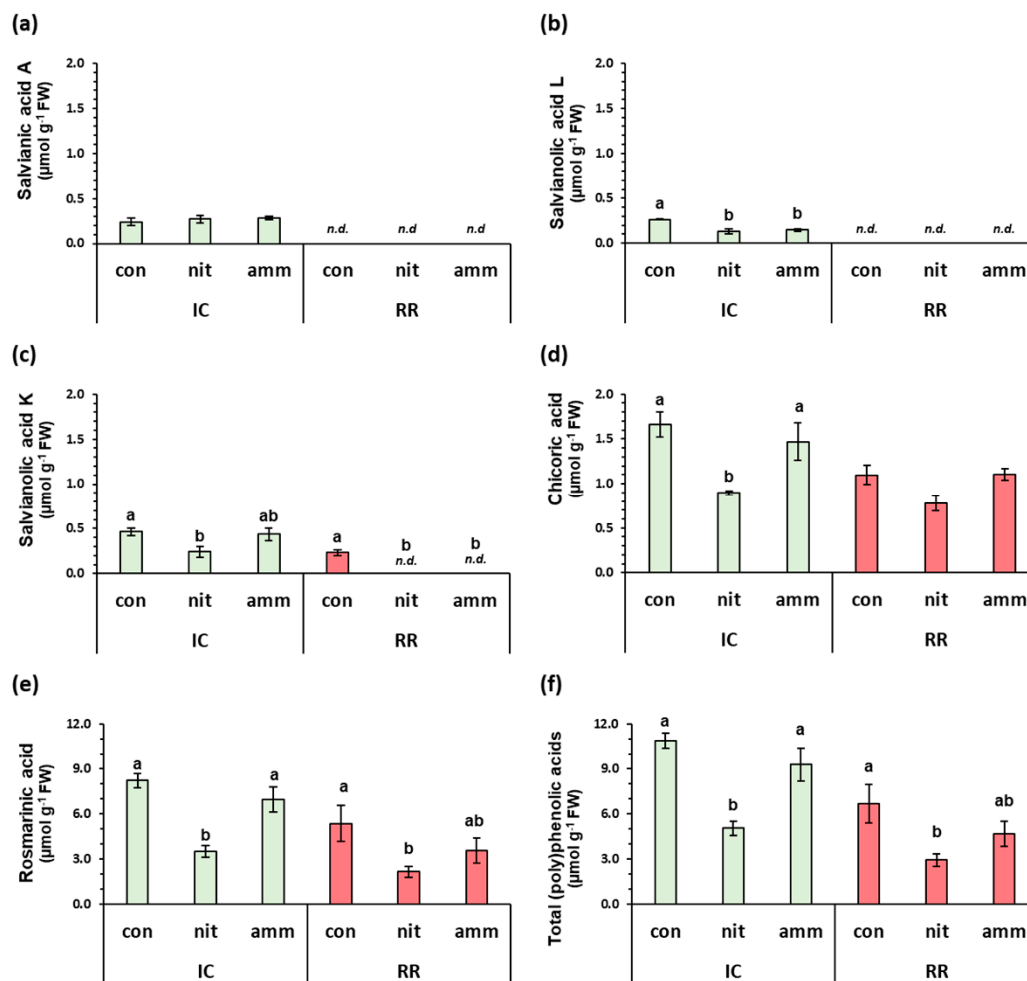


Figure 3. Contents of (poly)phenolic acids in basil leaves in the green ‘Italiano Classico’ (IC, green bars) and in the purple ‘Red Rubin’ (RR, red bars) cultivars. Plants were exposed for 5 d to absence of N—control [con], 10 mM NO_3^- —nitrate [nit], or 10 mM NH_4^+ —ammonium [amm]. Contents of: (a) salvianic acid A; (b) salvianolic acid L; (c) salvianolic acid K; (d) chicoric acid; (e) rosmarinic acid; (f) total (poly)phenolic acids, calculated as sum of the previous molecules. n.d.: not detected. Values are means \pm SEM (n = 3), expressed in $\mu\text{mol g}^{-1}$ FW. Results are expressed in two graph scales to visualize differences. Statistical significance was assessed by one-way ANOVA and Tukey’s test within each cultivar. Various letters indicate significant differences among the different nutritional treatments ($p < 0.05$).

3.5. Effects of Mineral N Forms on the Contents of Individual Flavonoids in Leaves of Purple Basil

The evaluation of the contents of individual flavonoids in leaves of green and purple basil exposed to the different N nutritional treatments was conducted by LC-ESI-MS/MS (see Supplementary Table S2 for technical details). In IC, this approach allowed to detect very low levels of quercetin rutinoside, which were not affected by the N nutritional treatments (data not shown). In RR, it was possible to quantify 11 compounds, including simple dihydroquercetin, quercetin, naringenin, apigenin, and cyanidin derivatives, as well as highly decorated anthocyanins. In particular, this last class of compounds comprised the most abundant molecules, such as anthocyanin A, anthocyanin C, and anthocyanin D2 (Figure 4a-m). Finally, the contents of total flavonoids were calculated as the sum of the individual 11 molecules detected (Figure 4n).

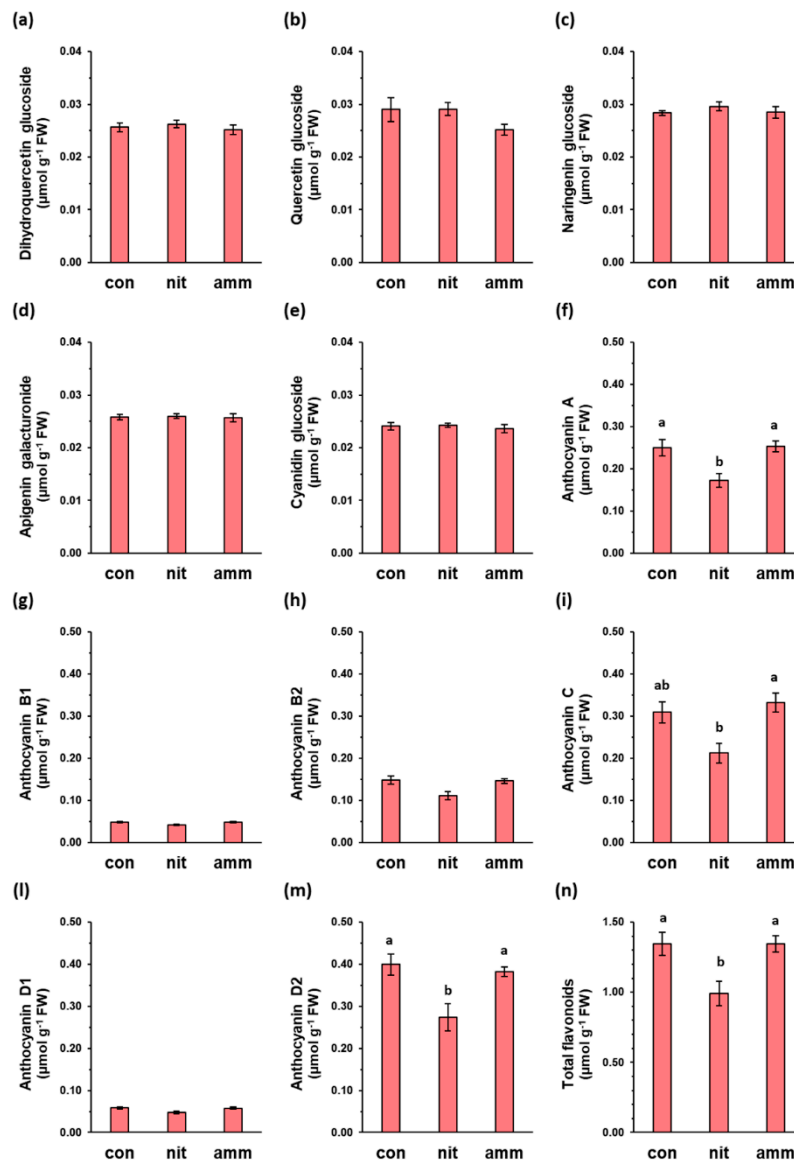


Figure 4. Contents of flavonoids in basil leaves in the purple cultivar ‘Red Rubin’. Plants were exposed for 5 d to absence of N—control [con], or to 10 mM NO₃[−]—nitrate [nit], or to 10 mM NH₄⁺—ammonium [amm]. Contents of: (a) dihydroquercetin glucoside; (b) quercetin glucoside; (c) naringenin glucoside; (d) apigenin galacturonide; (e) cyanidin glucoside; (f) anthocyanin A; (g) anthocyanin B1; (h) anthocyanin B2; (i) anthocyanin C; (l) anthocyanin D1; (m) anthocyanin D2; (n) contents of total flavonoids, calculated as the sum of the previous molecules. Values are means ± SEM (n = 3), expressed in µmol g^{−1} FW. Results are expressed in different graph scales to visualize differences. Statistical significances were assessed by one-way ANOVA and Tukey’s test. Various letters indicate significant differences among the different nutritional treatments (*p* < 0.05).

The classification above allowed to depict different effects of the N nutritional treatments. The contents of the less abundant flavonoids, like simple flavonoids (i.e., dihydroquercetin glucoside, quercetin glucoside, naringenin glucoside, apigenin galacturonide; Figure 4a–d), and of a few anthocyanins (i.e., cyanidin glucoside, anthocyanins B1 and B2, and anthocyanin D1; Figure 4e,g,h,l) did not change in response to the N nutritional treatments. Conversely, the levels of the more abundant (and highly acylated) anthocyanins dropped in plants exposed to NO₃[−] nutrition [nit], whereas they remained high in plants exposed to NH₄⁺ availability [amm] (+47% for anthocyanin A, +57% for anthocyanin C, +40% for anthocyanin D2 compared to NO₃[−]-fed plants, *p* = 0.031, *p* = 0.026, and

$p = 0.046$, respectively; Figure 4f,i,m), with concentrations similar to those measured in the controls [con]. Once again, this behavior was well mirrored by the results on the contents of total flavonoids (+35% in the NH_4^+ -fed plants compared to the NO_3^- -fed ones, $p = 0.040$, Figure 4n).

Overall, the results highlight that the supply of N as either NO_3^- or NH_4^+ exerted distinct effects on the accumulation of flavonoids in the studied model RR basil cultivar, and that specific sets of molecules were differently affected.

4. Discussion

Hydroponics is a soilless agricultural system that offers several advantages, including high productivity, low soil demand, minimal contamination and strict control of the fertilization inputs [16]. In particular, several studies report the high suitability of this technique for the improvement of crop yield and quality traits in basil production [14,15,17,19]. Although in small-scale, in this work we established an experimental hydroponic protocol that allowed to fulfill several purposes (Figure 1a). First of all, the protocol allowed to conduct experiments in controlled conditions starting from commercial young plants, previously grown in soil by traditional agronomic techniques. Secondly, the period of cultivation in low N condition allowed to obtain adult plants in vegetative phase (close to the harvest period [2]) characterized by high growth uniformity (Figure 1b) and able, after a short period of N starvation, to promptly respond to changes in N availability.

In this study, green basil ('Italiano Classico', IC) was characterized by higher biomass than the purple one ('Red Rubin', RR, Supplementary Table S1). This result is in agreement with other studies reporting that purple basil cultivars, including RR, show in the field lower plant height than the green ones [1], lower dry weight yield in large-scale hydroponic production systems [14], and, finally, that RR plants are taller than those of the green cultivar 'Tigullio' when grown in pots outdoors [41]. According to the literature [41], purple basil has lower biomass because part of the carbon reserves is diverted from growth to the biosynthesis of anthocyanins, a process that, although metabolically expensive, confers advantages against abiotic stresses. This different carbon allocation could also partly explain the higher L/R ratio observed in purple RR compared to green IC, which might be due to a lesser translocation of photosynthates to the root system (Supplementary Table S1).

In both the green and the purple cultivars considered in the present work, the availability of NO_3^- sustained the highest biomass production, in particular in the leaves, while NH_4^+ nutrition induced an increase of the L/R ratio, mainly affecting root growth (Figure 2). Kiferle et al. [19] reported that, in IC plants at blooming phase, the increase in NO_3^- concentration in the hydroponic solution led to an increase in shoot fresh weight and to a decrease in root biomass, whereas NH_4^+ had negative effects on both organs. The discrepancy with our results could depend on the different plant developmental phase (vegetative vs blooming), and, more likely, on the different duration of the N treatments. However, our observations are consistent with the observation that NO_3^- can promptly stimulate leaf expansion, by acting as an osmoticum and/or by sustaining the biosynthesis of cytokinins [21]. Moreover, negative effects on root growth in plants exposed to NH_4^+ as sole N form were described in several plant species [21,26].

The evaluation of the contents of NO_3^- , NH_4^+ , and total amino acids in roots and leaves allowed to verify that the N nutritional treatments adopted exerted significant effects on the plant N nutritional status in the two basil cultivars considered (Table 1). As expected, the exposure of plants to NO_3^- or to NH_4^+ sustained an increase in the contents of the respective ion in both IC and RR. Moreover, NH_4^+ nutrition induced the accumulation of amino acids in both roots and leaves, a typical response observed in several plant species [21,26,33]. In addition, considering that chlorosis is a typical symptom of prolonged N deficiency or excess NH_4^+ [24,26], the observation that the chlorophyll contents did not decrease in response to the N treatments (Table 2) indicates that both of these undesirable conditions were avoided in our experimental design. Finally, NO_3^- availability was also concomitant with a reduction in NH_4^+ levels, suggesting that the higher plant growth favored the consumption of the endogenous reserves of NH_4^+ (Figure 2 and Table 1).

The observation that the levels of NH_4^+ were different in IC and RR cultivars depending on the organ is interesting (Table 1). The use of NH_4^+ by plants requires a broad modulation of metabolism, including glycolysis, the oxidative pentose phosphate pathway and the tricarboxylic acid cycle, in order to provide C skeletons and reducing equivalents to the NH_4^+ assimilative pathway [26,42]. Moreover, the contribution of roots and leaves in NH_4^+ assimilation depends on species and cultivar, and influences the ability of the plant to adapt to this nutrient [43,44]. Interestingly, our results showed a different behavior of IC and RR. In particular, whereas in roots the levels of NH_4^+ were higher in IC than in RR, RR showed generally higher contents of NH_4^+ than IC in leaves. In addition, in the NH_4^+ -treatment, amino acid accumulation in RR leaves was significantly lower than in IC leaves, suggesting a lesser need of the purple basil cultivar to prevent hazardous accumulation of this potentially toxic cation. Concomitantly, RR leaves showed higher contents of chlorophyll (Table 2), consistent with what observed comparing cyanic and green plants of other basil cultivars and plant species [45]. Overall, the results suggest that purple basil cultivars may have a greater capability than the green ones to safely manage NH_4^+ in leaves, and allow to hypothesize that anthocyanins may contribute to reduce the potential NH_4^+ toxicity, similar to what observed for excess boron nutrition [28].

This hypothesis is also supported by the results regarding the antioxidant capacity and the metabolism of phenolic compounds in the leaves (Table 2). An overview of our results allows to confirm some evidences described in the literature. In particular, the leaf antioxidant capacity was very similar in IC and RR plants, but RR leaves showed higher levels of total phenols, most likely due to anthocyanin accumulation. Hence, in basil leaves, the antioxidant capacity appears to be very poorly correlated to the contents of total phenols and anthocyanins, as reported by several authors [5,46,47]. Moreover, our study confirms, in plants grown in hydroponic condition, that leaves of purple basil possess higher PAL activity than those of green basil (Table 2), consistent with what reported for in vitro cultured basil seedlings [31], and, by means of RNA-Sequencing analysis, for potted plants grown outdoors [45], suggesting that purple cultivars are able to maintain this trait in a broad range of growth conditions. Furthermore, the results about (poly)phenolic acids and flavonoids (Figure 3; Figure 4, Supplementary Table S2) are highly consistent with our recent description of the composition of bioactive molecules in IC and RR leaves [5]. The present work confirms that basil leaves contain, in addition to high levels of rosmarinic and chicoric acids, small amounts of salvianolic acids L and K. Considering the pharmacological properties of salvianolic acids [48], the presence of these compounds could contribute to enhance the nutraceutical value of basil. Similarly, this work confirms that the most abundant anthocyanins in purple basil leaves have a very high degree of decoration [5,49], that confers them peculiar technological properties and physiological functions [7]. However, in comparison to our previous work on plants of the same cultivars grown in pots in the greenhouse for 45 d (including germination) [5], in the present results the main difference resides in a reduction by about 20% in the content of total (poly)phenolic acids. Although small effects due to the plant growth stage could not be excluded, this observation confirms what reported in the literature [17], suggesting, on the basis of the defensive roles of these molecules [50], that in hydroponic conditions plants experience low environmental stress.

At the same time, the present study provides novel information about how, in basil, phenolic metabolism is influenced by N availability and by the type of N form (NO_3^- or NH_4^+ nutrition). One of the most interesting points is that this metabolism was differently affected in the green and in the purple cultivars considered (Table 2). Moreover, the results highlight that the content of each compound changed individually, suggesting that the different branches of the phenol biosynthetic pathway were specifically modulated (Figures 3 and 4).

The responses to NO_3^- nutrition were similar in the two basil cultivars, confirming that high availability of this nutrient is generally associated with a decrease in antioxidant capacity and total phenols in basil leaves (Table 2). In both cultivars, a decrease was observed in the contents of salvianolic acid K and rosmarinic acid, as well as, in IC only, in the contents of salvianolic acid L and chicoric acid

(Figure 3). According to several studies [18,19,31], this response is typically related to the hypothesis that, in plants overcoming N deficiency, a re-direction of C reserves from secondary metabolism towards biomass accumulation occurs. This consideration explains well the changes described at the level of the main polyphenolic compounds, like rosmarinic acid. Moreover, since salvianolic acids are downstream metabolites of rosmarinic acid [48], the decrease in their levels might be a secondary effect induced by the lower abundance of their precursor.

Nonetheless, our results also highlight that, when N is supplied to plants as NH_4^+ , a metabolic adaptation different than that occurring in nitric nutrition was evoked, and that the two cultivars responded in different ways to this nutrient. The plant responses to NH_4^+ are deeply affected by the plant nutritional status, with particular regard to the availability of NO_3^- [43]. However, the hydroponic experimental design adopted in the present study (Figure 1), which included a period of low N nutrition and one of N starvation, allowed to induce a decrease in the plant endogenous reserves of N (Table 1), useful to better distinguish the plant responses to NH_4^+ nutrition. In the green cultivar (IC), the main difference between the two inorganic N forms concerned the accumulation of specific polyphenolic acids (Figure 3). In particular, whereas NO_3^- nutrition led to a generalized decrease in the contents of these compounds, plants exposed to NH_4^+ showed higher levels of salvianolic acid K, chicoric acid, and, notably, of rosmarinic acid. Since these molecules act as ROS scavengers [3], it is conceivable that this response may contribute to protect leaf metabolism from the oxidative stress typically observed in NH_4^+ -fed plants [27]. These results, seemingly in contrast with other studies that report a decrease in the leaf contents of rosmarinic acid in basil plants exposed to NH_4^+ nutrition until bloom [19], may be explained by taking into account the different plant developmental phases considered in the present study.

In the purple RR cultivar, the main difference between NO_3^- and NH_4^+ nutrition regarded the anthocyanin contents, maintained at higher levels in the latter nutritional condition (Figure 4). Interestingly, the effect did not involve all of the flavonoids, but only highly decorated anthocyanins. This observation deserves particular consideration, especially because the different classes of flavonoids have specific physical-chemical characteristics. Simple flavonoids are well characterized for their antioxidant properties, whereas highly acylated anthocyanins are very effective in the absorption of visible and UV-B light and in photoprotection [41,51]. Hence, an intriguing hypothesis is that purple basil may respond to NH_4^+ , not only by an increase in phenols involved in the ROS-detoxifying systems, as observed in IC, but also by a modulation of light interception in the leaves. Overall, these results support a photoprotective role of anthocyanins during nutritional stresses able to affect photosynthesis, similarly to what observed in basil for boron toxicity [28] and in lettuce for N deficiency [29]. Further studies are needed to clarify this specific issue. On the other hand, it is also possible that the accumulation of anthocyanins could be beneficial by enhancing the capability for NH_4^+ sequestration into the vacuole, which would consequently reduce the requirement for boosting metabolic activities in order to accelerate the assimilation of the cation. Further studies, aimed to analyze the relationship among NH_4^+ inputs, light intensity, and timing of treatments, could provide novel cues about the physiological roles of highly decorated anthocyanins in basil.

5. Conclusions

Nitrogen is one of the main factors that affect plant growth and yield in most crops. This study provides new evidence that, although N fertilization is needed to sustain high yields, in green and purple basil the supply of NO_3^- could reduce the contents of important phenolic compounds, such as rosmarinic acid and anthocyanins. This study also reveals that the supply of NH_4^+ to plants in hydroponic cultivation could have lower impact than NO_3^- on the content of these compounds, suggesting that this nutritional strategy could be useful to enhance the technological and nutraceutical value of basil. Finally, the study highlights the importance of evaluating the contents of individual (poly)phenolic compounds to better assess the quality of this crop. Although further studies are needed to confirm in other cultivars the observed responses, estimate yield, and evaluate the feasibility of

large-scale hydroponic production, the present work provides new information about the relationship between N nutrition and phenolic metabolism in green and purple basil.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/4/498/s1>, Supplementary Material: Table S1: comparison of plant biomass in green and purple basil, Table S2: details about compound identifications by LC-ESI-MS/MS.

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