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Humic substances stimulate maize nitrogen assimilation and amino acid metabolism at physiological and molecular level

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Abstract

Background: The effects of a humic substance (HS) extracted from a volcanic soil on the nitrate assimilation pathway of *Zea mays* seedlings were thoroughly examined using physiological and molecular approaches. Plant growth, the amount of soluble proteins and amino acids, as well as the activities of the enzymes involved in nitrogen metabolism and Krebs cycle, were evaluated in response to different HS concentrations (0, 1 and 5 mg C L^{-1}) supplied to maize seedlings for 48 h. To better understand the HS action, the transcript accumulation of selected genes encoding enzymes involved in nitrogen assimilation and Krebs cycle was additionally evaluated in seedlings grown for 2 weeks under nitrogen (N) sufficient condition and N deprivation.

Results: HS at low concentration (1 mg C L^{-1}) positively influenced nitrate metabolism by increasing the content of soluble protein and amino acids synthesis. Furthermore, the activity and transcription of enzymes functioning in N assimilation and Krebs were significantly stimulated.

Conclusions: HS treatment influenced the gene expression of *Zea mays* plants at transcriptional level and this regulation was closely dependent on the availability of nitrate in the growth medium.

Keywords: Humic substances; Biological activity; Gene expression; Nitrogen metabolism

Background

Humic substances (HS) are the major components of soil organic matter (SOM) and positively affect crop production [1] by playing pivotal roles on both soil-plant system [2] and plant metabolism and development [3-6]. The growing concern for sustainable agriculture, whose goals are to ensure lower environmental costs and long-term productivity of agro-ecosystems [7], highlights the importance of developing management strategies to maintain and 1protect soil resources and, in particular, SOM [8]. Despite this present awareness, the second half of the past century has seen a massive and indiscriminate use of high-energy input in agriculture and in particular of N fertilizers during the so-called green revolution. These practices led to the selection of genotypes, which were highly productive when fed with high nitrogen inputs, though not efficient in nitrogen use [9], while they concomitantly caused a progressive impoverishment of soil physical and biological properties, as well as of SOM and nutrients' bioavailability.

Because of the importance of HS in soil fertility, a larger number of works focused on both the chemical-physical structure and the biological properties of humic matter. HS have been traditionally described as polydispersed heterogeneous organic compounds with large molecular weight [10]. However, recent experimental findings reached a new understanding of the humic conformational nature that is regarded as a supramolecular association of heterogeneous molecules with relatively low molecular weight (≤1.5 kD) held together in only apparently large molecular sizes by weak interactions, such as hydrogen and hydrophobic bondings. The metastable conformations of humic

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associations can be reversibly disrupted by interactions with small amounts of organic acids [11,12], while the same amount of acids do not alter conformation of the true macropolymers stabilized by covalent bonds [13]. The supramolecular nature of humic matter and its response to organic acids have been advocated to explain the bioactivity of humus on plants [6], its molecular dynamics [14] and the slow release of sorbed contaminants [15]. Furthermore, the recognition that NOM is composed by supramolecular associations rather than macropolymers has allowed the development of a fractionation strategy, called humeomics, that enables the analytical detection of most of the single molecules which constitute the supramolecular assembly [16-18]. These findings had profound implications on our understanding of SOM function and reactivity.

Several studies have reported the positive effect of HS on crop yield [19-21] and on root and shoot development [6,22]. In addition, leaf chlorophyll content [1], nutrient uptake [3,23-26] and the activities of enzymes involved in several physiological pathways, such as nitrogen assimilation [3,24,25,27] and energy metabolism [28-30], seem to be positively affected by HS. However, because of the complexity of HS' nature, the relationship between their molecular structure and biological activity is not yet completely clear. In the last decades, several studies correlated biological activity to humic chemical features [31-41]. The positive effects of different HS in relation to their chemical structure were shown by Muscolo et al. [35] and Nardi et al. [36] on *Pinus nigra* callus metabolism and *Zea mays* seedling growth. Moreover, Zancani et al. [42,43] successfully related humic matter molecular features to metabolism of tobacco BY-2 suspension cell cultures and to changes of cellular ATP and glucose-6-phosphate during embryogenesis of *Abies cephalonica*.

Furthermore, the complexity of the biological action exerted by HS on plant metabolism suggested the existence of hormonal mechanisms, in particular of an auxin-like effect [3,4]. The presence of low amount of IAA in different soil humic substances has been reported [33,44]. Moreover, these substances seem to have a physiological effect on both a maize isoform of H^+ ATPase *Mha2* [34,37,38], which is a specific auxin target, and a pea phospholipase A_2 [45], which is a component of an auxin-dependent signalling.

The objective of this work was to investigate the effect of humic matter on maize nitrate assimilation and amino acids metabolism when the humic concentration was varied. The response of growth, protein and free amino acid content of maize seedlings to humic supply was evaluated here. Moreover, the activity of the main enzymes involved in the Krebs cycle and nitrogen metabolism was determined, together with the expression of genes encoding the enzymes involved in these two metabolic pathways.

Methods

Soil humic matter extraction and chemical characterization

A humic acid (HA) from a Fulvudand soil of the volcanic caldera of Vico, near Rome, Italy, was isolated by standard methods as reported elsewhere [46]. The HA was titrated to pH 7.2 with a 0.5 M KOH solution in an automatic titrator (VIT 90 video titrator, Radiometer, Copenhagen, Denmark) under N_2 atmosphere and stirring. After reaching the constant pH 7.2, the solution containing potassium humate was left under titration for 2 h, filtered through a glass microfibre filter (Whatman GF/C; GE Healthcare, Buckinghamshire, UK) and freeze-dried. The elemental analyses and the molecular characterization of this HA by online thermochemolysis-GC-MS and CPMAS-NMR spectroscopy were reported earlier [36].

Plant material, growth solutions and HS treatment

Maize seeds (*Z. mays* L. var. DK 585) were soaked in distilled water for one night in running water and germinated for 60 h in the dark at 25°C on a filter paper wet with 1 mM $CaSO_4$ [47]. Then, the maize seedlings were raised in hydroponic conditions for 14 days in growth chamber with 450 mL of a complete nutrient solution (μM) containing KH_2PO_4 (40), $Ca(NO_3)_2$ (200), KNO_3 (200), $MgSO_4$ (200), FeNaEDTA (10), H_3BO_3 (4.6), $CuCl_2{\cdot}2H_2O$ (0.036), $MnCl_2{\cdot}4H_2O$ (0.9), $ZnCl_2$ (0.09) and $NaMoO_4{\cdot}2H_2O$ (0.01). The solution was renewed every 48 h. Growth chamber conditions were the following: day/night period of 14/10 h, air temperature of 27°C/21°C, relative humidity of 6%0/80% and photon flux density (PFD) of 280 μmol m^{-2} s^{-1}.

To evaluate morphometric parameters, protein and free amino acids content and enzyme activities, on the 12th day of growth, the plants were treated for 48 h with aqueous solutions in which the freeze-dried potassium humate was dissolved at different concentrations expressed as carbon weight per litre: 0 (control), 1 and 5 mg C L^{-1}.

To evaluate the transcript accumulation, the HS were applied on the 13th day at 1 mg C L^{-1} for 24 h to two different control solutions: a complete nutrient solution (see above) and a nutrient solution N-deprived, where N sources KNO_3 and $Ca(NO_3)_2$ $4H_2O$ were replaced with KCl and $CaCl_2{\cdot}2H_2O$ at the same concentrations. The expression analyses were performed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) on cDNA obtained from leaves and root tissues.

Determination of protein content

For the extraction of soluble protein, frozen foliar and root tissue (0.5 g) were ground in liquid nitrogen, stirred with the extraction buffer (100 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA, 5 mM DTT) and centrifuged at 14,000 g. The supernatants were mixed with 10% (*w*/*v*) trichloroacetic acid (TCA) and centrifuged. The pellets

obtained were then re-suspended in 0.1 N NaOH. The amount of total proteins in the leaves was determined by using the Bradford method [48] with bovine serum albumin as a standard. The results were expressed as mg protein/gramme fresh tissue.

Enzyme extraction and assay conditions

The enzymes were solubilized from leaves by manually crashing vegetal tissues in a mortar added with a 100 mM Hepes-NaOH solution at pH 7.5, a 5 mM $MgCl_2$ solution, and a 1 mM dithiothreitol (DTT) solution. The ratio of plant material to mixture solution was 1:3 *w*/*v*. The extract was filtered through two layers of muslin and clarified by centrifugation at 20,000 g for 15 min. The supernatant was used for enzymatic analysis. All steps were carefully performed at 4°C, while a Jasco V-530 UV-vis spectrophotometer (Jasco Co., Midrand, Gauteng, South Africa) was used for measuring enzyme activity.

The nitrate reductase (NR EC 1.7.1.1) activity was assayed according to Lewis and collaborators [49] and expressed as unit per milligramme protein. One unit corresponds to the production of 1 μmol of NO_2^- per minute at 25°C.

The nitrite reductase (NiR EC 1.7.1.4) activity was determined on the basis of the drop in NO_2^- concentration in the reaction medium [50]. After incubation at 30°C for 30 min, the NO_2^- content was determined colorimetrically at A_{540} and the activity was expressed as unit per milligramme protein. One unit corresponds to the consumption of 1 μmol of NO_2^- per minute at 25°C.

To evaluate the glutamine synthetase (GS EC 6.3.1.2) activity, the mixture for the assay contained 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM Na_2KAsO_4, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine and the appropriate amount of enzyme extract. The assay was performed in a final volume of 750 μL. The enzymatic reaction was developed for 15 min at 37°C. γ-Glutamyl hydroxamate (gh) was colorimetrically determined by addition of 250 μL of a mixture (1:1:1) of 10% (*w*/*v*) $FeCl_3.6H_2O$ in 0.2 M HCl, 24% (*w*/*v*) trichloroacetic acid and 50% (*w*/*v*) HCl. The optical density was recorded at A_{540} [51]. The activity was expressed as unit per milligramme protein. One unit corresponds to the production of 1 μmol mg protein per minute at 37°C.

NADH-dependent glutamate synthase (NADH-GOGAT EC 1.4.1.14) assay contained 25 mM Hepes-NaOH (pH 7.5), 2 mM L^{-1} glutamine, 1 mM α-ketoglutaric acid, 0.1 mM β-NADH, 1 mM Na_2 EDTA and 100 μL of enzyme extract in a final volume of 1.1 mL. GOGAT was assayed spectrophotometrically by monitoring β-NADH oxidation at A_{340}. The activity was expressed as unit per milligramme protein. One unit corresponds to the oxidation of 1 μmol of β-NADH per minute at 37°C.

The activity of aspartate aminotransferase (AspAT EC 2.6.1.1) was measured spectrophotometrically by monitoring β-NADH oxidation at A_{340} at 30°C. The assay medium (final volume 2.4 mL) contained 100 mM Tris-HCl at pH 7.8, 240 mM L-aspartate (Asp), 0.11 mM pyridoxal phosphate, 0.16 mM NADH, 0.93 kU malate dehydrogenase (MDH), 0.42 kU lactate dehydrogenase (LDH), 12 mM 2-oxoglutarate and 200 μL enzyme extract [52]. The activity was expressed as unit per milligramme protein. One unit corresponds to the oxidation of 1 μmol of β-NADH per minute at 37°C.

The activity of asparagine synthetase (AS EC 6.3.5.4) was determined by incubating 10 mM glutamine (Gln), 30 mM ATP, 10 mM Asp, 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, Hepes 50 mM pH 7.75 with 400 μL of the desalted extract in a total volume of 500 μL at 30°C for 60 min. The reaction was terminated with 100 μL of sulfosalicylic acid (200 mg mL^{-1}), centrifuged and an aliquot of the supernatant, adjusted to pH 7.0 with NaOH and taken for the analysis and separation of Asp and Asn by HPLC of the ortho-phthalaldehyde (OPA) reagent derivates [53]. The activity was expressed as unit per milligramme protein. One unit corresponds to the production of 1 μmol of Asn per minute at 30°C.

For the assay of citrate synthase (CS EC 2.3.3.1), the leaves were homogenized using 100 mM Tris-HCl buffer pH 8.2, containing 5 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 1 mM Na_2EDTA and 10% glycerol. The leaves were filtered and centrifuged as reported [54]. All steps were performed at 4°C. The CS enzyme was assayed adding 50 mL of oxalacetic acid 0.17 mM, 50 mL acetyl-CoA 0.2 mM, and 10 mL extract, to 3 mL of Tris-HCl 0.1 M (pH 8.0). This activity was measured spectrophotometrically at 25°C, by monitoring the reduction of acetyl-coenzyme A (acetyl-CoA) to CoA, at wavelength A_{232}. The activity was expressed as unit per milligramme protein. One unit corresponds to the reduction of 1 μmol of acetyl-CoA per minute at 30°C.

To extract $NADP^+$-isocitrate dehydrogenase ($NADP^+$-IDH EC 1.1.1.42) and MDH (EC 1.1.1.37), the leaves were homogenized using 100 mM Tris-HCl buffer pH 8.2 containing 5 mM β-mercaptoethanol (Sigma), 1 mM Na_2EDTA and 10% glycerol. The extracts were filtered and centrifuged as reported [54]. All steps were performed at 4°C.

For $NADP^+$-IDH activity, 50 μL of crude extract was added to 2.85 final volume of a reaction mixture containing 88 mM imidazole buffer (pH 8.0), 3.5 mM $MgCl_2$, 0.41 mM β-NADP-Na salt and 0.55 mM isocitrate-Na salt. The assay was performed at 25°C following the formation of NADP(H) at A_{340} [55]. The activity was expressed as unit per milligramme protein. One unit corresponds to the production of 1 μmol of NADP(H) per minute.

For MDH activity, 3.17 mL of assay mixture contained 94.6 mM phosphate buffer (pH 6.7), 0.2 mM β-NADH-Na_2 salt, 0.5 mM oxalacetic acid, and 1.67 mM $MgCl_2$. MDH activity was assayed at 25°C, following the formation of NAD^+ at A_{340} [54]. The activity was expressed as unit per milligramme protein. One unit corresponds to the production of 1 μmol of NAD^+ per minute.

Free amino acids determination

The free amino acids were analyzed, as by the method of Seebauer et al. [56]. Fifty milligrammes of homogenous dry powder was extracted for 1 h at room temperature with 1.5 mL of a 5% (*w*/*v*) TCA solution. The sample was clarified by centrifugation, and 1.5 mL of the supernatant was analyzed for free amino acids. The amino acid analysis was accomplished by precolumn OPA derivatization of the sample followed by reverse phase separation, using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a thermo-controlled auto-sampler, fluorescence detector and an Agilent HP Chemstation for data elaboration. The chromatographic conditions were described previously [57].

RNA extraction and cDNA synthesis

The tissue samples were ground in liquid nitrogen. Aliquots of approximately 100 mg were used for RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was quantified by spectrophotometric reading, and the quality was assayed by agarose gel electrophoresis. After DNAse treatment (Promega, Milan, Italy), first-strand cDNA was synthesized from 5 μg of total RNA using 200 U of MMLV Reverse Transcriptase (Promega, Milan, Italy) and oligo(dT) as a primer in 20 μL reactions, as described by Sambrook and collaborators [58].

Semi-quantitative RT-PCR analysis

To determine the expression level of *NR* (NCBI accession number M27821), *AS* (NCBI accession number X82849), *MDH* (NCBI accession number T27564), *CS* (NCBI accession number W49861) and *IDH* (NCBI accession number W21690), semi-quantitative RT-PCR experiments with specific primers were performed as described earlier [59]. Genes analyzed and relative primers are listed in Table 1. The constitutively expressed 18S rRNA (NCBI; Accession Number U42796.1) gene was used as an internal control of RNA quantity. PCR products were separated by electrophoresis in a 1-2.5% agarose gel stained with ethidium bromide, and quantified through the ImageJ program (ImageJ 1.38 J, Wayne Rasband, National Institute of Health, Bethesda, MD, USA). PCR reactions were performed on cDNAs obtained from two different RNA extractions from independent experiments and repeated at least three times for each cDNA. PCR products were further purified by using the QIAquick gel extraction Kit (Qiagen, Hilden, Germany) and sequenced, according to Sanger et al. [60], using the ABIPRISM original Rhodamine Terminator kit (Applied Biosystems, Branchburg, NJ, USA). The sequence comparisons were performed by using Blastx and Blastn computer programs (NCBI, National Center for Biotechnology Information).

Statistical analysis

All sets of experiments were repeated three times, and for each set three replicates were assayed. Data were the means of three replicates, and the standard deviations did not exceed 5%. The results obtained were processed statistically by the Student-Newman-Keuls test [61].

Results

Effects of HS treatments on plant growth

After growing for 12 days in a complete nutrient solution, the plants were transferred in a solution containing two different concentrations of HS and treated for 48 h. For seedlings supplied with 1 mg C L^{-1} of humic solution, no significant difference from control was found for leaves, roots length and fresh weight. Conversely, treatment with a greater humic concentration (5 mg C L^{-1}) produced length of both leaves and roots 8% shorter than the control seedlings (Figure 1a,b) and fresh weight (Figure 2a,b).

Effects of HS treatments on protein content and enzyme activities

The amount of proteins was always significantly lower in roots than in leaves of maize seedlings. Seedlings treated with 1 mg C L^{-1} of HS showed a leaf content of protein larger than control (7%). On the other hand, a significant decrease in protein content (–21%) was observed in the leaves of seedlings supplied with 5 mg C L^{-1} HS (Figure 3a). In roots, no significant differences with respect to the control were detected at 1 mg C L^{-1} of HS and the 5 mg C L^{-1} treatment even caused a 5% decrement in protein content (Figure 3b).

The humic addition to the growing medium affected the activities of NR and NiR in a concentration-dependent manner (Figure 4a,b). As for NR, the application of 1 and 5 mg C L^{-1} concentrations increased the NR specific activity by about 10% and 56%, respectively (Figure 4a). A similar behaviour was found for NiR activity. In plants supplied with 1 and 5 mg C L^{-1}, the NiR activity was increased by 12% and 41%, respectively (Figure 4b).

The GS and NADH-GOGAT enzymes showed a dose-response activity similar to those described for NR and NiR. When maize seedlings were supplied with HS at 1 and 5 mg C L^{-1}, GS and GOGAT activities increased by 11% and 30%, and by 64% and 92% respectively (Figure 5a,b).

Table 1 Specific forward and reverse primer sequences used in semi-quantitative RT-PCR

Gene	Primer forward (5′-3′)	Primer reverse (5′-3′)	Accession number
NR (nitrate reductase)	5′-TTCATGAACACTACCGACGTCG-3′	5′-GAGCCTGTACGGATACTCGGC-3′	M27821
AS (asparagine synthetase)	5′-CATCATTGAGCTCTCGCGCAGGTTAC-3′	5′-GGGGGAAATGTTATGAAGCGTTCACAA-3′	X82849
CS (citrate synthetase)	5′-GTTTGGTCATGGAGTTCTGCGTAA-3′	5′-GGAGGTACAACTTCATACAACTTGGACAC-3′	W49861
IDH (isocitrate dehydrogenase)	5′-AAACTCGAGGCTGCTTGCGTTGAGA-3′	5′-ATAATTAGCTTGCATCGAAACTGCGG-3′	W21690
MDH (malate dehydrogenase)	5′-GCCAGATTTCTGAGAGACTTAATGTCCA-3′	5′-TCGAGGCATGAGTAAGCAAGCGTCTT-3′	T27564
18S	5′-CCATCCCTCCGTAGTTAGCTTCT-3′	5′-CCTGTCGGCCAAGGCTATATAC-3′	U42796.1

As for AspAT, the plants supplied with 1 and 5 mg C L^{-1} of HS showed an increase in enzyme activity of 16% and 20%, respectively (Figure 6a). Conversely, the AS activity had a different trend, since 1 mg C L^{-1} caused a decrease in the AS activity (−15%) and 5 mg C L^{-1} of HS caused a significant larger stimulation (+18%) compared to the control (Figure 6b).

We evaluated the effects of HS on CS, $NADP^+$-IDH and MDH of maize seedlings because of their important role in maintaining the balance between C and N metabolism. When plants were supplied with HS at 1 and 5 mg C L^{-1}, an increase of CS activity by 56% and 98%, respectively, was observed in comparison to the control (Figure 7a). A similar trend was also observed for $NADP^+$-IDH and MDH activities (Figure 7b,c). The seedlings treated with 1 and 5 mg C L^{-1} showed an increase of both MDH and $NADP^+$-IDH activities by 14% and 20%, and 93% and 81%, respectively.

Effect of HS treatment on the amino acid content

The free amino acid content in leaves was measured to follow HS effects on nitrogen assimilation. Aspartate (Asp), threonine (Thr), isoleucine (Ile) and lysine (Lys) increased by 10% to 13% with 1 mg C L^{-1} HS, while the 5 mg C L^{-1} did not change the amino acids' content (Table 2). However, asparagine (Asn) behaved differently, as it was increased (41%) only with the addition of 5 mg C L^{-1} HS, while 1 mg C L^{-1} HS decreased (−11%) this amino acid in leaves as compared to control.

Effect of humic sample on gene expression

To gain further insight on the mechanisms of regulation of the enzyme previously described by HS, the expression of five genes encoding the key enzymes of N assimilation and organic acids synthesis was evaluated in leaves of both nitrate-supplied and nitrate-depleted maize seedlings, in response to 1 mg C L^{-1} HS for 24 h. The transcript

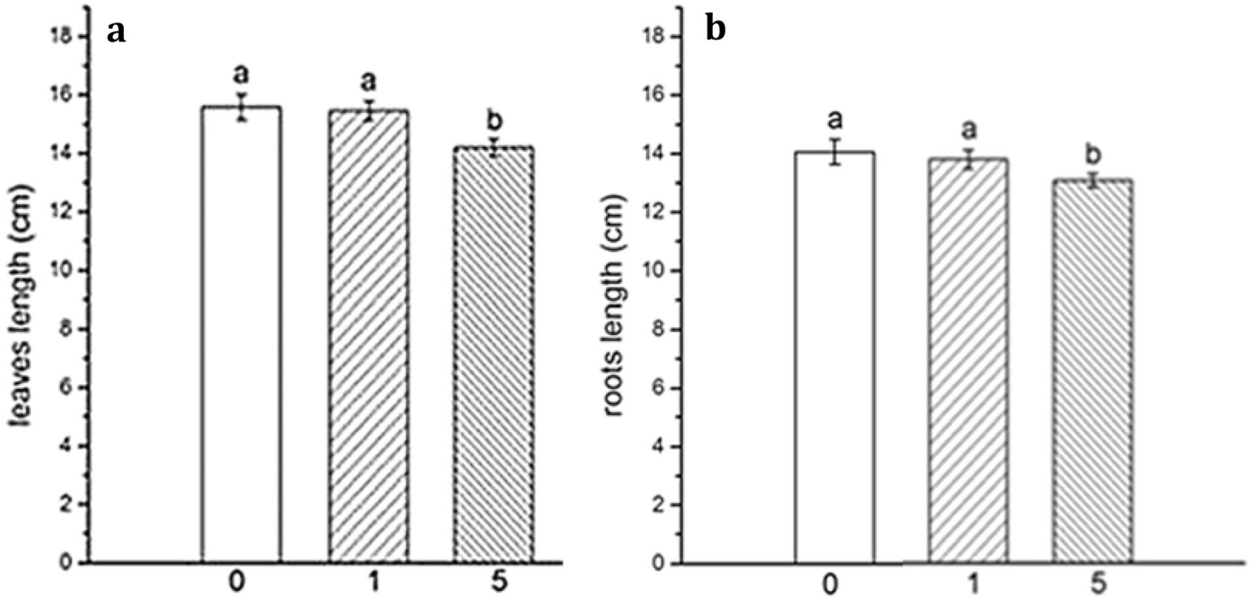

Figure 1 Length (cm) of leaves (a) and roots (b). Seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The length of roots and leaves was expressed in centimetres. Values are means of three independent determinations (±SE). Letters above bars indicates significant differences ($P < 0.05$).

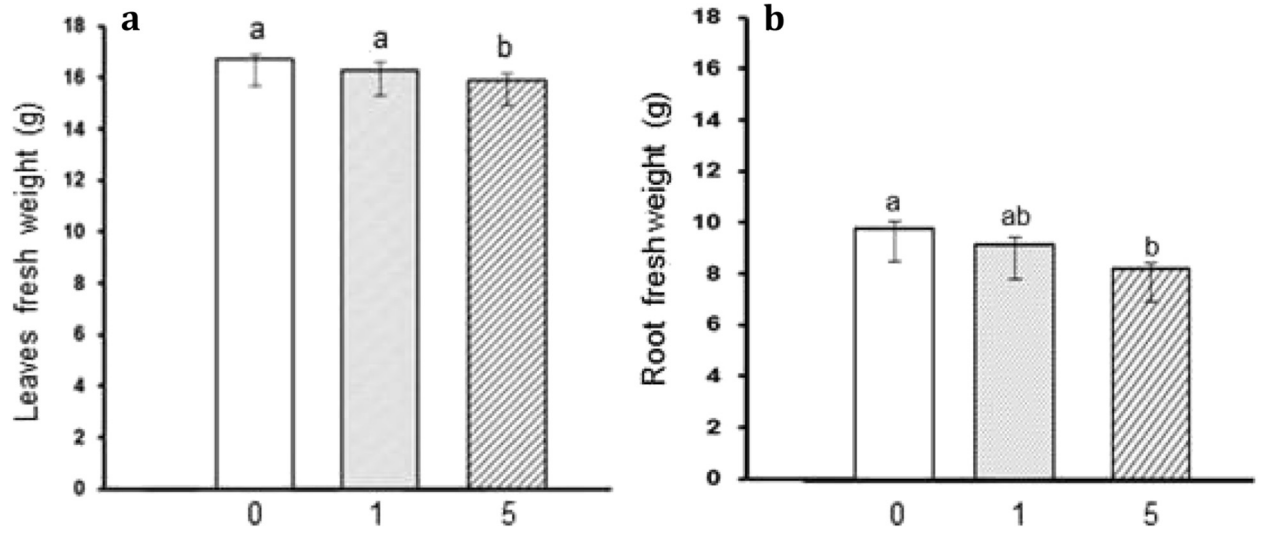

Figure 2 Fresh weight (g) of leaves (a) and roots (b). Seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The fresh weight was expressed in g. Values are means of three independent determinations (±SE). Letters above bars indicate significant differences ($P < 0.05$).

accumulation of the gene encoding a NADH-NR [59] showed a significant increase after the treatment with 1 mg C L^{-1} HS in leaves of N-supplied plants, with respect to that detected in leaves of +N control plants (Figure 8). Conversely, no NR transcript accumulation was observed in the leaves of N-deprived plants of either HS-treated or control seedlings. Similarly, humic treatment induced a substantial increase of mRNA abundance in leaves of N-supplied seedlings, whereas no significant differences were detected when N-depleted seedlings were supplied with HS.

A similar expression pattern in response to humic substances provision was also observed for the genes encoding MDH, CS and IDH enzymes which are involved in the Krebs cycle and N organication. In the case of MDH, a slight decrease of mRNA abundance was found in N-deprived leaves after humic supply. Moreover, the transcript

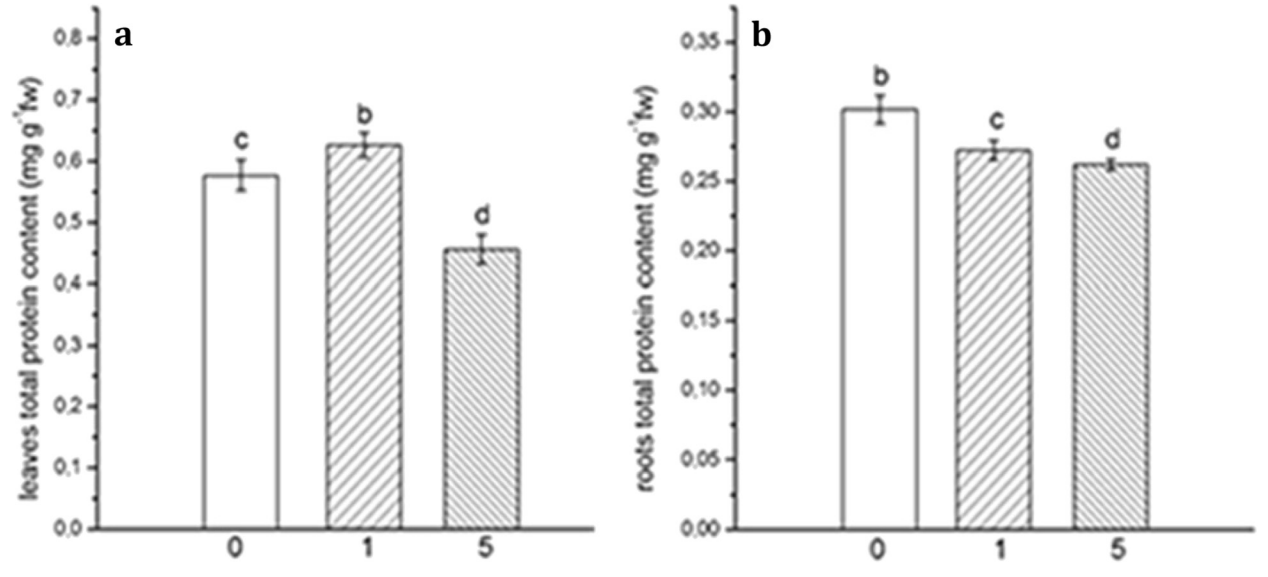

Figure 3 Total protein content in leaves (a) and roots (b). Seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The protein concentration was expressed in milligrammes of protein per gramme of fresh weight. Values are means of three independent determinations (±SE). Letters above bars indicates significant differences ($P < 0.05$).

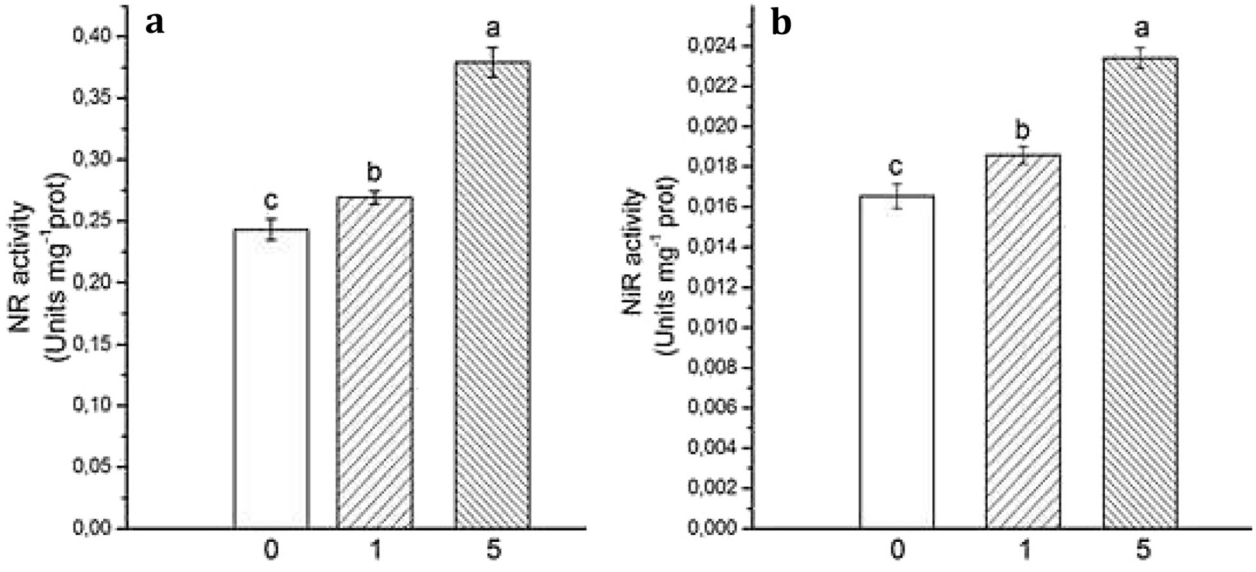

Figure 4 NR (a) and NiR (b) specific activities. Seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The specific activities were expressed in unit per milligramme protein. One unit corresponds to the production (NR) or consumption (NiR) of 1 μmol of NO_2^- per minute at the assay conditions. Values are the means of three independent determinations (±SE). Letters above bars indicates significant differences ($P < 0.05$).

level of these three genes was always lower in N deficiency conditions in respect to that for N-supplied plants.

Discussion

Since the 1980s, several studies have highlighted the existence of biological effects of HS on plant physiology and metabolism [1,3,6,25,32]. However, only more recently, the attention of researchers has been focused on HS fractions characterized by an apparently low-molecular-weight (LMW), which seems to influence plant growth and physiology more than other fractions [3,63]. In fact, several works focused on effects of LMW fraction on

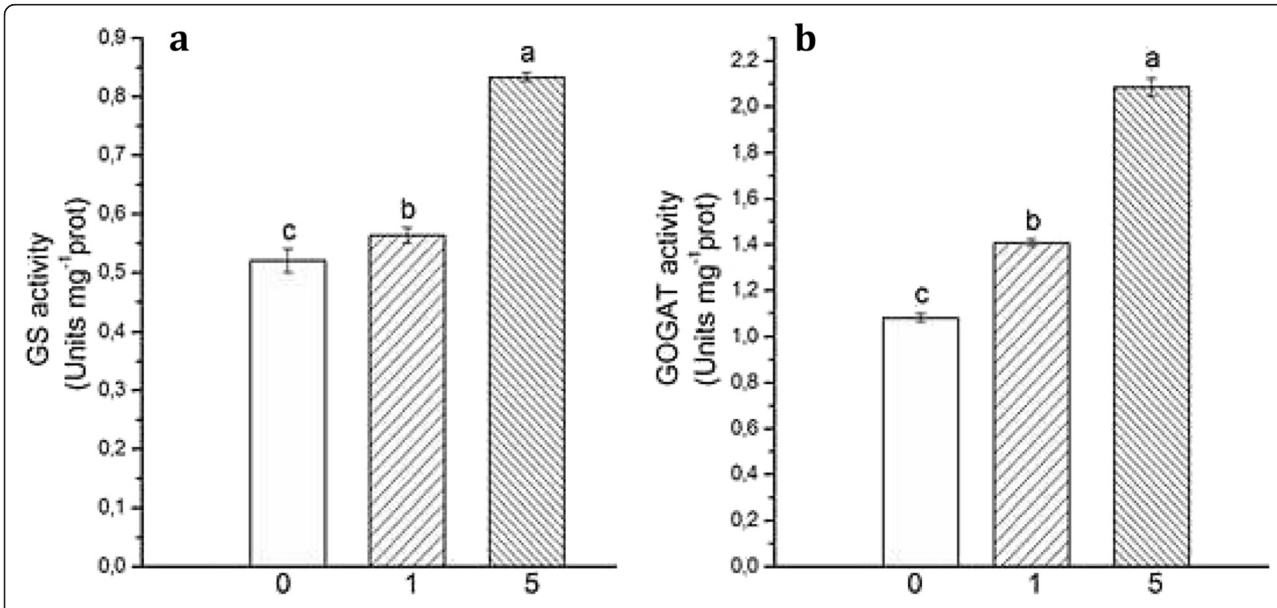

Figure 5 GS (a) and NADH-GOGAT (b) specific activities. Seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The specific activities were expressed as unit per milligramme protein. One unit corresponds to the production of 1 μmol of γ-glutamyl hydroxamate per minute in the case of GS and to the oxidation of 1 μmol of β-NADH per minute in the case of GOGAT at the assay conditions. Values are means of three independent determinations (±SE). Letters above bars indicate significant differences ($P < 0.05$).

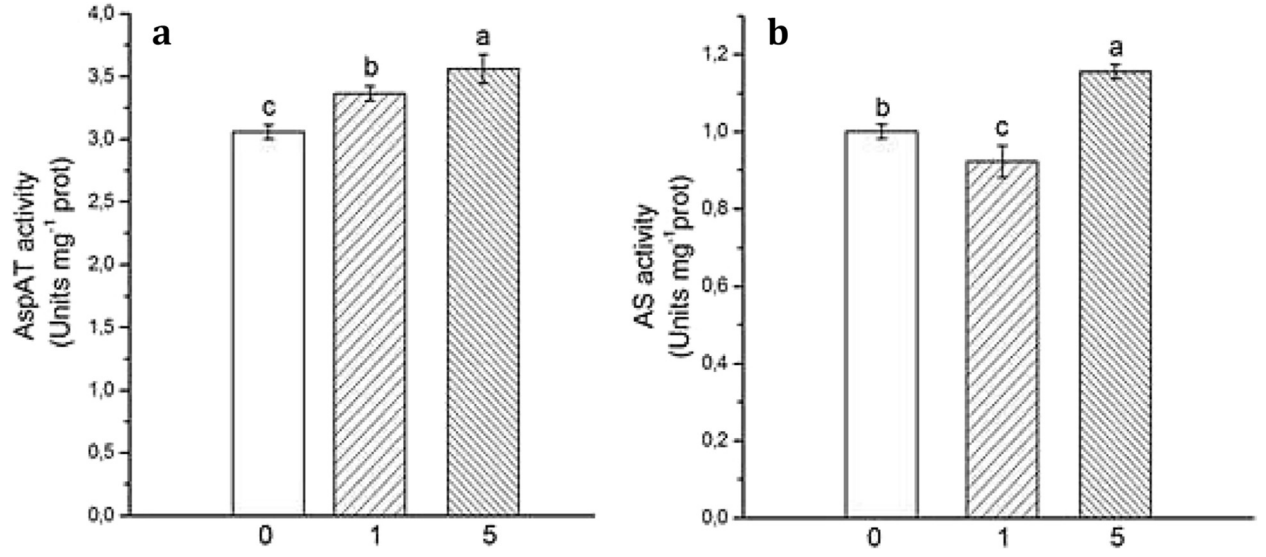

Figure 6 AspAT (a) and AS (b) specific activities. The seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The AspAT and AS specific activities were expressed in as unit per milligramme protein. For AspAT activity, 1 U corresponds to the oxidation of 1 μmol of β-NADH per minute at the assay conditions. For AS, 1 U corresponds to 1 μmol of Asn produced per minute at the assay conditions. Values are means of three independent determinations (±SE). Letters above bars indicate significant differences ($P < 0.05$).

nitrogen uptake [33,34,37,64] and assimilation [65], but the results obtained were often contradictory [66,67]. Their positive effects have mainly been attributed to a large content of carbohydratic and carboxyl-C groups [32,68].

More recently, Nardi and coworkers [36] evaluated the effect on the respiratory metabolism of maize seedlings provided by different size fractions of a soil humic acid, after their separation by preparative high-performance size-exclusion chromatography (HPSEC). The evaluation of the biological activity of size fractions showed that the size fraction with the smallest molecular size was more bioactive, followed by, in the order of, both the original bulk humic acid and the larger size fractions. Such effect was attributed to the larger flexibility of the smallest size fraction conferred by its greater content of hydrophilic components [36]. In fact, both the conformational flexibility and the hydrophilicity of this small-sized humic fraction should facilitate the release from its supramolecular structure upon the action of organic acids exuded by roots of humic molecules with plant stimulation activity. The diffusion of such bioactive molecules in solution should be less easy from humic matrices with larger and more compact conformations [37,38,69]. Here, we used the same bulk humic acid that was found bioactive and second only to its separated smallest size fractions in order to evaluate its effects on nitrogen assimilation in maize at both physiological and molecular levels. This species was chosen because of its worldwide economic and agronomic importance and also because of its importance as a model plant [70].

Our results indicate that the HS treatment influenced the activities of all these enzymes in a dose-dependent manner. In particular, the increase of enzyme activity in relation to the dose of HS supplied was exponential as a significant stimulation was observed when plants were treated with 5 mg C L^{-1}. A different trend was observed for AS, because its specific activity was strongly stimulated only in response to the 5 mg C L^{-1} humic solution, while it was slightly inhibited by the 1 mg C L^{-1} treatment.

The synthesis of Asn is in competition with that of Asp, Lys, Thr and Ile, which represent, together with the amino acids of the aspartate family [71], the main substrates for the synthesis of structural proteins and enzymes required for the optimal plant development [72]. However, Asn may also represent a transitory store of N to be later used to meet specific demands during plant growth [73]. Our results showed a significantly higher accumulation of Lys, Ile and Thr in leaves of seedlings grown with 1 mg C L^{-1} of HS in comparison to those observed in control plants that is in accordance with the higher content of soluble proteins measured in HS-treated leaves.

To deepen the understanding of molecular events underlying the HS effects on plant physiology, the expression of five genes encoding the enzymes involved in few key steps of N assimilation was evaluated. To discriminate N-dependent from N-independent molecular effects of HS, the transcript amount was measured in leaves of both nitrate-supplied and nitrate-depleted HS-treated seedlings. In this case, the choice of a 24-h treatment was aimed at evaluating the earlier molecular effects of

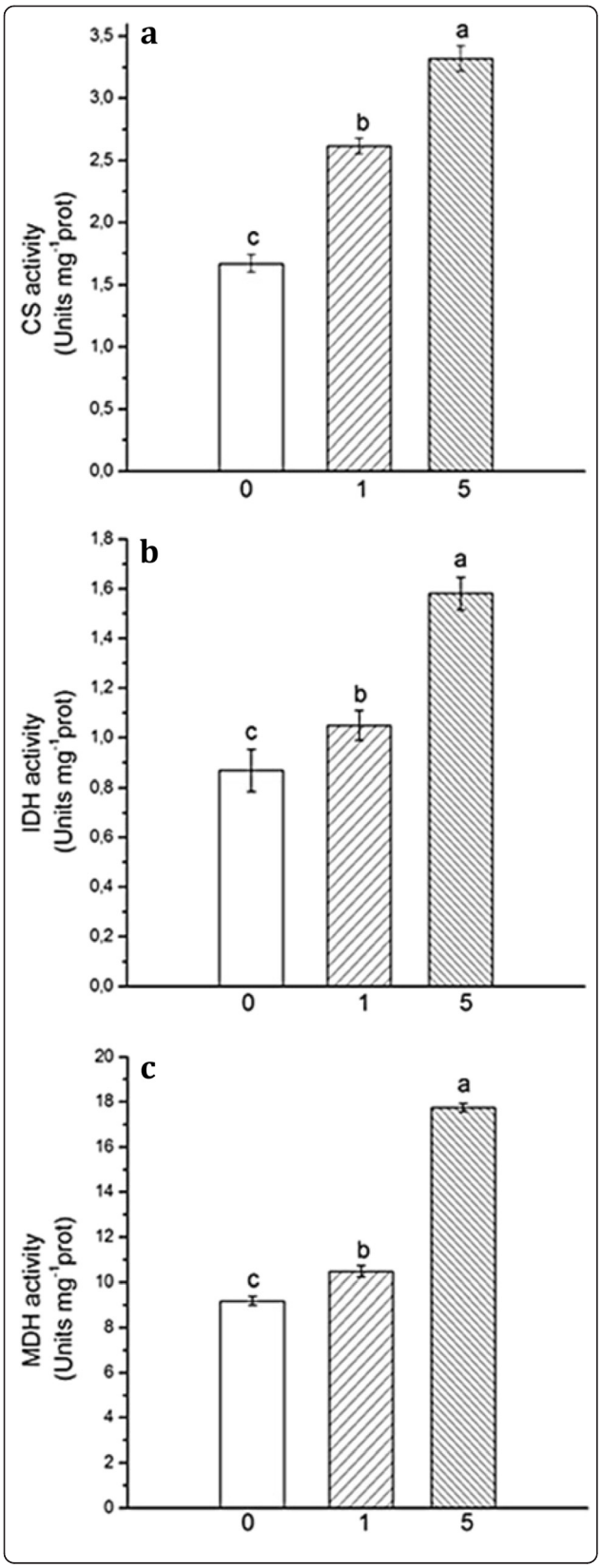

Figure 7 CS (a), NADP$^+$-IDH (b) and MDH (c) specific activities. The seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. The specific activities were expressed unit per milligramme protein. For CS, 1 U corresponds to the reduction of 1 μmol Ac-CoA per minute; for NADP$^+$-IDH, 1 U corresponds to 1 μmol of NADPH reduced per minute and for MDH to 1 μmol of NAD^+ formed per minute at assay conditions. Values are means of three independent determinations (±SE). Letters above bars indicate significant differences ($P < 0.05$).

humic matter. In fact, Trevisan et al. [40] showed that the gene transcription in response to HS is regulated already after a few hours of treatment.

Our findings show an increase of mRNA abundance of all genes therein studied upon HS treatment only when seedlings were grown in the presence of nitrate. On the contrary, HS did not induce any increase of transcript accumulation when the nutrient solution was depleted of nitrate. These results globally suggest that the enzyme stimulation observed after the humic supply may be generally regulated at the transcriptional level. An exception seems to be represented by the expression of the AS gene that was strongly induced after 24 h of treatment with 1 mg C L^{-1} solution, whereas the AS activity measured after 48 h was lower than for the control. This may be due either to a more rapid down-regulation of the AS activity in response to the duration of HS treatment or otherwise to the occurrence of some post-transcriptional regulatory mechanisms. Furthermore, since most of the genes analyzed are nitrate-responsive, it may be hypothesized that the HS regulation of the transcription of genes involved in nitrogen metabolism may be mediated by a nitrate-dependent signal, since HS' stimulatory effect is evident only when the nutrient solution contained nitrate.

Conclusions

Our results indicate a positive dose-dependent effect of the humic acid used here on the activities of the main enzymes involved in the reduction and assimilation of inorganic nitrogen. The enhanced bioactivity of HS may

Table 2 Amino acid contents (μmol g^{-1} fw) in leaves

Amino acid	Treatment		
	C	1	5
Aspartate	1.74 ± 0.3	1.97 ± 0.52	1.8 ± 0.45
Threonine	2.78 ± 0.92	3.06 ± 0.96	2.76 ± 0.92
Isoleucine	1.31 ± 0.27	1.46 ± 0.35	1.3 ± 0.62
Lysine	1.36 ± 0.32	1.53 ± 0.47	1.3 ± 0.31
Asparagine	1.7 ± 0.4	1.55 ± 0.18	2.4 ± 0.39
Glutamate	2.85 ± 0.90	3.19 ± 0.96	2.71 ± 0.52

Seedlings were grown for 14 days in a Hoagland modified complete nutrient solution (0) and treated for the last 2 days with HS concentrations of 0, 1 and 5 mg C L^{-1}. Mean values ± SD were obtained from five independent measurements.

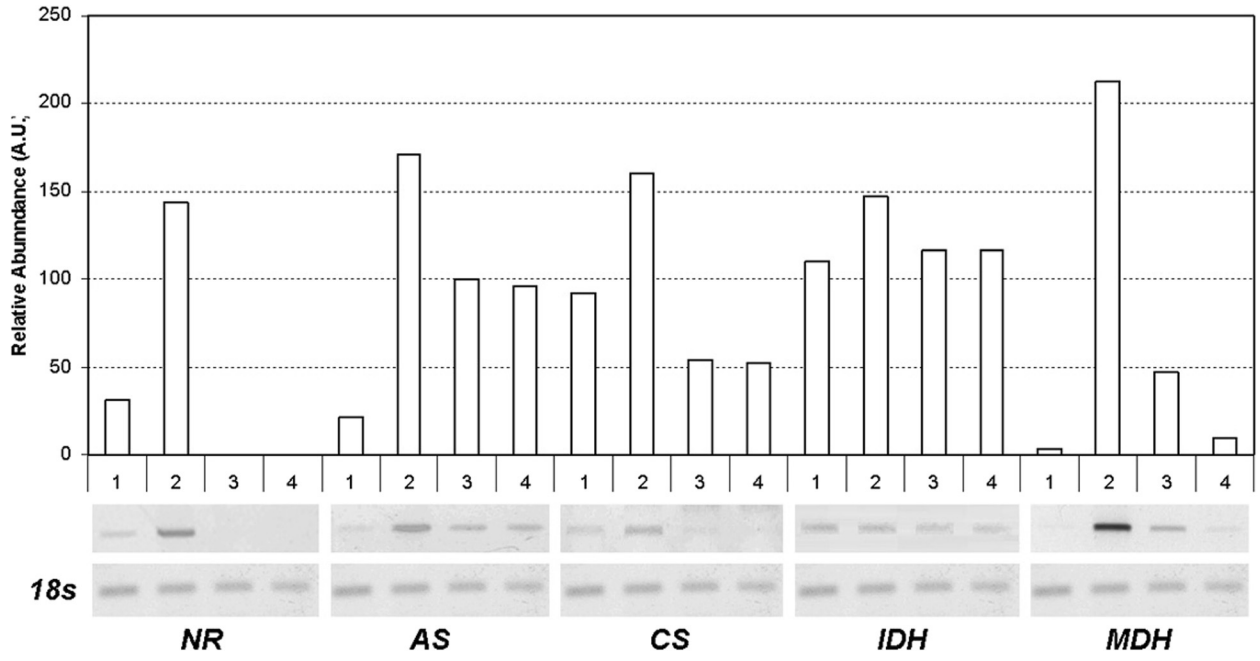

Figure 8 Analysis of the expression of NR, AS, CS, IDH and MDH in leaves. The measurements were performed by using semi-quantitative RT-PCR method in the linear range. The seedlings were grown in a control complete nutrient solution (1) and in a control N-deprived solution (3) for 14 days. One milligramme C L−1 of HS was added for 24 h after 13 days to both +N (2) and −N solutions (4). The specific transcript levels in the *y*-axis of histograms (top panel) are normalized against that of the reference gene and expressed in arbitrary units (AU).

be explained with the tendency to increase the size of supramolecular aggregation of humic molecules with concentration [74] and the concomitant enhanced capacity to provide specific bioactive molecules to targeted root cell membranes [6].

Among all enzymes, only the AS showed a different trend of regulation in response to HS, possibly because of a competition of its own substrate with the biosynthesis of amino acids of the Asp family [75], thus favouring the accumulation of a large amount of Asn in seedling leaves treated with the greatest HS concentration. This is not surprising considering that Asn accumulation in young leaves is an indirect result of the restriction of protein synthesis when the metabolism is subjected to stress conditions [73]. While further studies should be certainly conducted to increase our understanding on the dose-response of HS, molecular findings suggest that the biochemical response to humic treatments may principally depend on a transcriptional mechanism of regulation, as previously hypothesized for the regulation of a maize isoform of H^+-ATPase [34] and with a more holistic approach by Trevisan et al. [46]. However, the induction of a gene expression in response to HS seems to depend closely on the presence of nitrate in the growing medium because humic matter alone was not able to up-regulate the transcription of genes. These findings suggest that HS may act as an additional signal in the regulation of gene expression mediated by nitrate, by either promoting its bioavailability or interfering with the signalling pathway that leads plants to adapt their metabolism to the nutrient availability [64,76].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SV carried out the molecular genetic studies and participated in drafting the manuscript. AE participated in drafting the manuscript and made the corrections. AN participated in drafting the manuscript. AM, AP and SN conceived of the study, participated in its design and coordination and helped draft the manuscript. SQ carried out the molecular genetic studies and participated in drafting the manuscript. All authors read and approved the final manuscript.

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