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Research article

The root application of a purified leonardite humic acid modifies the transcriptional regulation of the main physiological root responses to Fe deficiency in Fe-sufficient cucumber plants

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ABSTRACT

The aim of this study is to investigate the effect of a well-characterized purified humic acid (non-measurable concentrations of the main plant hormones were detected) on the transcriptional regulation of the principal molecular agents involved in iron assimilation. To this end, non-deficient cucumber plants were treated with different concentrations of a purified humic acid (PHA) (2, 5, 100 and 250 mg of organic carbon L^{-1}) and harvested 4, 24, 48, 76 and 92 h from the onset of the treatment. At harvest times, the mRNA transcript accumulation of CsFRO1 encoding for Fe(III) chelate-reductase (EC 1.16.1.7); CsHa1 and CsHa2 encoding for plasma membrane H^+-ATPase (EC 3.6.3.6); and CsIRT1 encoding for Fe(II) high-affinity transporter, was quantified by real-time RT-PCR. Meanwhile, the respective enzyme activity of the Fe(III) chelate-reductase and plasma membrane H^+-ATPase was also investigated.

The results obtained indicated that PHA root treatments affected the regulation of the expression of the studied genes, but this effect was transient and differed (up-regulation or down-regulation) depending on the genes studied. Thus, principally the higher doses of PHA caused a transient increase in the expression of the CsHa2 isoform for 24 and 48 h whereas the CsHa1 isoform was unaffected or down-regulated. These effects were accompanied by an increase in the plasma membrane H^+-ATPase activity for 4, 48 and 96 h. Likewise, PHA root treatments (principally the higher doses) up-regulated CsFRO1 and CsIRT1 expression for 48 and 72 h; whereas these genes were down-regulated by PHA for 96 h. These effects were associated with an increase in the Fe(III) chelate-reductase activity for 72 h. These effects were not associated with a significant decrease in the Fe root or leaf concentrations, although an eventual effect on the Fe root assimilation pattern cannot be ruled out.

These results stress the close relationships between the effects of humic substances on plant development and iron nutrition. However, further studies are needed in order to elucidate if these effects at molecular level are caused by mechanisms involving hormone-like actions and/or nutritional factors.

1. Introduction

The beneficial effect of humic substances (HS) on the growth of different plant species has been directly related to significant improvements in Fe and Zn plant uptake [1,2]. Likewise, a number of studies have reported that the capacity of different metal–humic complexes to affect the growth of plants cultivated in different soils was principally linked to the ability of these complexes to supply available micronutrients [1,3]. Other studies also indicate that the way in which HS act on plant development includes specific effects on the activity of certain enzymes involved in nutrient assimilation and more specifically in iron root uptake by "strategy I" plants growing under Fe-deficient conditions [4].

Abbreviations: ABA, abscisic acid; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; FRO, ferric chelate-reductase; GA3, GA4, and GA7, gibberellins A3, 4 and 7; HPSEC, high-performance size-exclusion chromatography; HS, humic substances; IAA, indole-3-acetic acid; IHSS LHA, IHSS reference leonardite humic acid; IHSS, International Humic Substances Society; iP, isopentenyladenine; iPR, isopentenyladenosine; IRT, iron-regulated transporter; MRM, multiple-reaction monitoring; MW, molecular weight; PHA, purified humic acid; PM, plasma membrane; WEHS, water extracted humic substances; Z, zeatin; t-ZR and c-ZR, *trans*- and *cis*-zeatin riboside.

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It is very well known that in order to alleviate Fe deficiency, plants develop specific mechanisms oriented to increase both Fe availability in the rhizosphere and the metabolic efficiency of Fe within the plant [5,6]. This specific response seems to differ according to the plant species [5]. "Strategy I" plants (non-graminaceous monocot and dicot) develop an ensemble of root responses such as (i) the release of protons by plasma membrane H^+-ATPase (EC 3.6.3.6) into the rhizosphere to increase iron solubility; (ii) the induction of both a plasma membrane-bound Fe(III) chelate-reductase (EC 1.16.1.7) to reduce Fe(III) to Fe(II), and specific high-affinity Fe(II) transporters, such as IRT1, to facilitate the transport of Fe(II) into root cells; (iii) the development of sub-apical swelling with abundant root hairs, transfer cells; and finally, (iv) the release of organic molecules with reducing and complexing capacity [5–9].

In this context, the capacity of a specific fraction of HS (water extracted fraction WEHS) to increase some of the plant responses evolved under Fe deficiency conditions, such as Fe(III) chelate-reductase activity, has been reported in cucumber [4]. This action of WEHS was also linked to a significant increase in rhizosphere acidification [4].

In principle this action of HS may be directly affecting the biochemical processes involved in Fe-stress responses at molecular level, or indirectly, affecting these processes when previously activated by Fe deficiency. If the action of HS is direct, a measurable activation at molecular level of the processes involved in Fe-stress responses could be expressed in Fe-sufficient plants, while if this action is mainly indirect, it would be principally expressed under Fe-deficient conditions.

As mentioned above, previous studies have demonstrated the capacity of HS to affect Fe-stress mechanisms in the roots of Fe-starved plants [4]. We have not found studies in the literature, however, that deal with both the HS effects on root Fe uptake mechanisms in plants growing under Fe-sufficient nutrition, and the effects of these substances on the gene expression of the Fe(III) chelate-reductase and the iron transporter IRT1.

Therefore, in order to study the direct or indirect character of the action of HS on Fe-stress responses in strategy I plants, we investigated the effect of different concentrations of a well-characterized and purified humic acid on the transcriptional expression and activity of the main molecular agents involved in root Fe uptake – the root plasma membrane H^+-ATPase, the Fe(III) chelate-reductase and the Fe(II) transporter IRT1 – in cucumber plants growing under Fe-sufficient conditions.

2. Materials and methods

2.1. Humic acid extraction and purification

A humic acid obtained from leonardite (PHA) was employed in the experiments. The PHA was extracted and purified using the IHSS methodology as described elsewhere [10]. Briefly, 10 g of non-dried leonardite were mixed with 200 mL of 0.1 M NaOH in a 250-mL flask. After 48 h stirring at 25 °C in darkness, the supernatant containing the unfractionated humic extract was separated from the solid fraction by centrifugation at 7650 *g* for 30 min. The extraction was carried out under inert atmosphere (N_2). The PHA was obtained by acidifying an aliquot of the alkaline extract containing the humic acids with hydrochloric acid (HCl) 6 M up to a pH of 1.5. After 12 h, the acidified sample was centrifuged at 7650 *g* for 30 min in order to separate the precipitated humic acid from the supernatant containing the fulvic acids and other acid-soluble organic compounds. After washing with water to eliminate Cl^- contamination, PHA was freeze-dried.

2.2. Physicochemical characterization of PHA

The main physicochemical features of PHA were characterized using elemental analysis, potentiometric analysis, ^{13}C NMR and high-performance size-exclusion chromatography (HPSEC), as described below. In order to study the representativeness of PHA as a model humic acid, its properties were compared with those of a reference leonardite humic acid (IHSS LHA) obtained from the International Humic Substances Society (IHSS).

2.2.1. Elemental analysis

The carbon, hydrogen and nitrogen concentrations of the lyophilized samples were analyzed in duplicate by a LECO CHN 900 analyser. The oxygen concentration was calculated by difference.

2.2.2. Potentiometric analysis

Stock solutions of 0.1% (w/w) of PHA and IHSS LHA were prepared separately by dissolving an adequate amount of the lyophilized molecular system in 0.1 M NaOH. Once the molecular system had been dissolved, adequate amounts of an H^+-cationic exchange resin (Amberlite IRA-118H^+) were added to the stock solution to attain a final pH of 3.5. The resin was separated by centrifugation (15 min at 5000 *g*) followed by filtration (Whatman no. 42).

In order to carry out the titration studies, an aliquot of the stock solution corresponding to 27 mg of the molecular system was added to a water solution containing 0.5 mL of 0.1 M $HClO_4$ and the necessary volumes of 1 M KNO_3 to achieve an ionic strength of 0.1 M. The final volume was 35 mL. The solution was titrated with 0.05 mL increments of 0.1 M NaOH using a Metrohm Titrando 809 and the pH was measured by means of a Metrohm combined pH glass electrode (Metrohm 6.0258.000). To ensure that the equilibrium between measurements has been reached, non-increment in the base addition was made until the pH measure varied under 0.01 pH unit over 5 min. Titrations were carried out under N_2 atmosphere. All titrations were carried out in duplicate.

The experimental data was analyzed for functional group concentrations using the procedure designed by Takamatsu and Yoshida [11] involving the use of the Gran plot method to calculate the different equivalent points corresponding to each mean class of acidic groups (strong acidity), weak acidity-carboxylic groups and very weak acidity-phenol groups.

2.2.3. ^{13}C NMR spectroscopy

^{13}C NMR spectra were obtained on a Varian Unity 300 spectrometer at 75.429 MHz using the cross-polarization magic angle spinning technique (CPMAS), with a spinning speed of 5 kHz, 90° pulse width, 69 ms acquisition time and 1.0 s delay.

The HPSEC study: the PHA and IHSS LHA molecular size distributions were obtained using HPSEC. The chromatographic system consisted of a Waters 600 Controller pump followed by two detectors in series: a Waters 996 Photodiode Array Detector set at 400 nm, and a Waters 2424 Refractive Index Detector (RI). Size-exclusion separation occurred through a PL Aquagel-OH 30 column (Polymer Laboratories), preceded by a guard column with the same stationary phase.

For each sample, solutions of 800 mg L^{-1} of organic carbon were prepared in 0.05 M $NaNO_3$. The injection volume of all samples was 100 μL, the eluent used was 0.05 M $NaNO_3$ (pH 7), and the flow rate was 1 mL/min. Void volume ($V_0 = 6.65$ mL) and permeation volume ($V_p = 11.82$ mL) were determined with polyethylene oxide of molecular weight (MW) of 43,250 Da and methanol, respectively.

In order to evaluate an approximate MW distribution from HPSEC chromatograms for PHA and IHSS LHA, a universal calibration was

carried out using polyethylene glycol and polyethylene oxide standards of known MW as described in Baigorri et al. [12].

2.3. Analysis of the concentration of plant regulators in PHA

The concentration of the principal plant regulators in PHA was analyzed using HPLC/MS/MS as described below.

The following hormones were studied: zeatin (Z), dihydrozeatin (DHZ), *trans*- and *cis*-zeatin riboside (t-ZR and c-ZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellins A 3, 4 and 7 (GA3, GA4, GA7). The extraction and purification of the different plant regulators were carried out using the method described by Dobrev and Kamínek [13], with some variations.

2.3.1. Extraction and purification procedures for cytokinins

Frozen PHA (0.5 g) (previously triturated in a mortar to a powder with liquid nitrogen) was homogenized with 5 mL of precooled (−20 °C) methanol–water–formic acid (15:4:1, v/v/v). Deuterium-labelled CK internal standards (2H5t-Z, 2H5t-ZR, 2H6-iP, 2H6-iPR, Olchemim, Olomouc, Czech Republic) were added (20 μL of a stock solution of 50 ng mL^{-1} of each standard in methanol) to the extraction medium. After overnight extraction at −20 °C, solids were separated by centrifugation (12,000 rpm, 10 min, 4 °C) and re-extracted for 1 h with an additional 4 mL of extraction mixture. Supernatants were passed through a Sep-Pak Plus tC18 cartridge (ref. WAT054925, Waters Co., Milford, MA) preconditioned with 2 mL of methanol followed by 2 mL of extraction medium. After evaporation to near dryness using a Labconco Vortex Evaporator, the residue was re-dissolved in 2 mL of 1 M formic acid, and applied to an Oasis MCX column (ref. 186000254, Waters Co., Milford, MA) preconditioned with 2 mL of methanol and 2 mL of 1 M formic acid. The column was washed successively with 2 mL of 1 M formic acid, 2 mL of methanol and 2 mL of 0.35 M NH_4OH, and the CK bases and ribosides were eluted with 2 mL of 0.35 M NH_4OH in 60% (v/v) methanol. This eluted fraction was evaporated to dryness in the vortex evaporator and re-dissolved in 200 μL of methanol–0.05% formic acid (30:70, v/v) before its injection in the LC/MS/MS system.

2.3.2. Liquid chromatography–mass spectrometry quantification of CKs

The CKs were quantified by HPLC linked to a 3200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using reverse-phase column (Tracer Excel 120 ODSA 3 μm, 100 × 4.6 mm, Teknokroma, Barcelona, Spain). A linear gradient of methanol (A) and 0.05% formic acid in water (B) was used: 35–95% A in 11 min, 95% A for 3 min and 95–35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.25 mL/min, the injection volume was 50 μL and the column and sample temperatures were 30 and 20 °C, respectively.

Detection and quantification were performed by multiple-reaction monitoring (MRM) in the positive-ion mode, employing a multilevel calibration graph with deuterated CKs as internal standards. The monitored fragmentation processes are listed in Table 1.

The source parameters are curtain gas: 25.0 psi, GS1: 50.0 psi, GS2: 60.0 psi, ion spray voltage: 5000 V, CAD gas: medium, and temperature: 600 °C.

2.3.3. Extraction and purification procedures for IAA, ABA and GAs

Frozen PHA (0.5 g) (previously triturated in a mortar to a powder with liquid nitrogen) was homogenized with 5 mL of precooled (−20 °C) methanol–water (80:20, v/v). Deuterium-labelled internal standards (2H5IAA and 2H6ABA, Olchemim, Olomouc, Czech Republic) were added (40 μL of a stock solution of 200 ng/mL of each standard in methanol) to the extraction medium. After overnight extraction at −20 °C, solids were separated by centrifugation (12,000 rpm, 10 min, 4 °C) and re-extracted for 1 h with an additional 4 mL of extraction mixture. Supernatants were passed through a Sep-Pak Plus tC18 cartridge (ref. WAT054925, Waters Co., Milford, MA) preconditioned with 2 mL of methanol followed by 2 mL of extraction medium. After evaporation to near dryness using a vortex evaporator, the hormones were extracted with two portions of 5 and 4 mL of diethyl ether, and afterwards the organic phase was evaporated. The residue was re-dissolved in 200 μL of methanol–0.5% acetic acid (30:70, v/v) before its injection in the LC/MS/MS system.

Table 1
Optimal parameters for the MRM detection of CKs in the LC-ESI–MS/MS analysis.

Compound	Transition	Dwell (ms)	DP	EP	CEP	CE	CXP
Z	220.3 > 136.2	150	36	3.5	16	25	4
DHZ	222.3 > 136.2	150	41	3.0	16	29	4
tZR	352.3 > 220.2	150	46	4.5	19	25	4
cZR	352.3 > 220.2	150	46	4.5	19	25	4
DHZR	354.3 > 222.2	150	36	4.0	19	27	4
iP	204.3 > 136.2	150	31	4.0	14	21	4
iPR	336.2 > 204.2	150	36	4.0	18	23	4
$^2H_5$t-Z	225.1 > 136.9	150	31	3.5	16	27	4
$^2H_5$t-ZR	357.1 > 225.2	150	31	6.0	20	25	4
$^2H_6$-iP	210.2 > 136.9	150	31	5.0	14	21	4
$^2H_6$-iPR	342.2 > 210.2	150	61	4.5	20	25	4

Abbreviations: DP, de-clustering potential; EP, entrance energy; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

2.3.4. Liquid chromatography–mass spectrometry quantification of IAA, ABA and GAs

The hormones were quantified by HPLC linked to a 3200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using an reverse-phase column (Synergi 4μ Hydro-RP 80A, 150 × 2 mm, Phenomenex, Torrance, CA). A linear gradient of methanol (A) and 0.5% acetic acid in water (B) was used: 35% A for 1 min, 35–95% A in 9 min, 95% A for 4 min and 95–35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 mL/min, the injection volume was 50 μL and the column and sample temperatures were 30 and 20 °C, respectively.

The detection and quantification were performed by MRM in the negative-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standards. The monitored fragmentation processes are listed in Table 2.

The source parameters are curtain gas: 25.0 psi, GS1: 50.0 psi, GS2: 60.0 psi, ion spray voltage: −4000 V, CAD gas: medium, and temperature: 600 °C.

2.4. Plant experiments

2.4.1. Experiment 1

This involves the validation study of the primers used to analyze CsFRO1, CsIRT1 and CsHa1 and CsHa2, RNA transcripts in cucumber roots.

Table 2
Optimal parameters for the MRM detection of IAA, ABA and GAs in the LC-ESI–MS/MS analysis.

Compound	Transition	Dwell (ms)	DP	EP	CEP	CE	CXP
IAA	174.0 > 130.1	150	−30	−3.0	−12	−14	−4
ABA	263.1 > 153.1	150	−25	−3.5	−20	−16	−4
GA_3	345.1 > 143.1	150	−50	−4.0	−20	−36	−4
GA_4	331.1 > 257.2	200	−55	−7.0	−16	−28	−5
GA_7	329.1 > 223.2	200	−45	−5.0	−18	−26	−5
$^2H_5$IAA	179.0 > 135.2	150	−20	−7.0	−12	−14	−4
$^2H_6$ABA	269.1 > 159.2	150	−25	−12	−14	−14	−4

Abbreviations: DP, de-clustering potential; EP, entrance energy; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

Seeds of *Cucumis sativus* L., (cv *Ashley*) were germinated in perlite irrigated with a solution containing 1 mM of $CaSO_4$. During the 2 weeks following post-germination, the plants were irrigated with an aerated 1/3-strength complete nutrient solution containing 1 μM of Fe (Fe–EDTA). The nutrient solution contained 2 mM $Ca(NO_3)_2$; 0.75 mM K_2SO_4; 0.65 mM $MgSO_4$; 0.5 mM KH_2PO_4, 50 μM KCl; 10 μM H_3BO_3; 1 μM $MnSO_4$; 0.5 μM $CuSO_4$; 0.5 μM $ZnSO_4$ and 0.35 μM Na_2MoO_4 [14]. The following week the different treatments commenced: plants were separated into two groups corresponding to the two different treatments, one without Fe (−Fe) and the other with 40 μM of Fe (+Fe). After 24, 48 and 72 h from the onset of the treatments the plants were harvested and the gene expression corresponding to the Ferric (Fe(III) chelate-reductase (CsFRO1)), H^+-ATPase (CsHa1 and CsHa2) and Fe(II)-transporter (CsIRT1) were studied. The number of replications for each measurement was of three per treatment. The experiment has been carried out three times.

The plants were cultivated in a growth chamber (irradiance: 250 μmol m^{-2} s^{-1}) at a photoperiod of 15/9 h day/night, an average temperature of 23/21 °C day/night and a relative humidity of 60–75%.

2.4.2. Experiment 2

This involves the time-course study on the effect of PHA root application on the expression in the root of the genes encoding Fe(III) chelate-reductase (CsFRO1), H^+-ATPase (CsHa1 and CsHa2) and Fe(II) transporter (CsIRT1); the activities in the root of Fe(III) chelate-reductase, and H^+-ATPase; and the concentration of Fe in leaves and roots.

Seeds of *C. sativus* L., (cv *Ashley*) were germinated in darkness on filter paper moistened with $CaSO_4$ 1 mM. Seven-day-old seedlings were transplanted to hydroponics medium in 900-mL dark glass vessels containing aerated nutrient solution as described above (Section 2.4.1) [14]. The nutrient solution contained 40 μM of iron as EDTA chelate. The pH of the nutrient solution was held at 6 in order to avoid the decomposition of Fe–EDTA chelate and Fe precipitation. Likewise, the non-appearance of significant interaction of EDTA chelated-Fe with PHA was probed by the absence of any quenching effect of chelated-Fe of the main PHA fluorescence peaks (data not shown). No precipitation of Fe inorganic species was observed throughout the experiment. The plants were grown in a greenhouse at 25/15 °C day/night, 40–60% of humidity and natural daylight for a photoperiod of 14 h. After 21 days of plant growth the following treatments were carried out: a control treatment that only received the nutrient solution and four PHA treatments that received the nutrient solution and four different concentrations of PHA expressed as organic carbon (C): 2, 5, 100 and 250 mg L^{-1}. The nutrient solution contained 40 μM of iron as EDTA chelate in all cases. After 4, 24, 48, 72 and 96 h from the onset of PHA treatments, the plants were harvested and the plant material was used for the different analytical determinations. Forty-eight hours before each harvest, the plants were stabilized in a growth chamber (irradiance: 250 μmol m^{-2} s^{-1}) at a photoperiod of 14/10 h day/night, an average temperature of 23/21 °C day/night and a relative humidity of 60–75%. Likewise, the plants received 6 h of light before harvest.

All determinations were carried out employing three replications. The experiment was carried out twice.

2.5. Iron concentration in the shoots and roots of control and PHA-treated cucumber plants

The Fe concentration in both the roots and the leaves of control and PHA-treated cucumber plants were analyzed in samples corresponding to 4, 24, 48, 72 and 96 h after the addition of PHA, previous microwave acid sample digestion (8 mL of concentrated HNO_3 and 2 mL of H_2O_2 for 0.5 g dry sample). The Fe concentration was measured by ICP-OES (Thermo Elemental Co. Iris Intrepid II XDL).

2.6. Real-time RT-PCR analysis of mRNA transcript

Apical roots (2–3 cm) were collected and disrupted with liquid nitrogen prior RNA extraction. Total RNA was extracted from 60 mg of crushed root using 350 μL guanidine–HCl lysis buffer of Nucleospin RNA plant kit (Macherey-Nagel, Germany) and 3.5 μL of β-mercaptoethanol with a rotor-stator for 45 s following the manufacturer's instructions. The DNase-treated RNA was quantified by measuring the absorbance of the pooled elution at 260 nm. The RNA quality was confirmed as a 260/280 nm ratio above 1.8. The integrity of RNA was verified by electrophoresis on 1% formaldehyde agarose gels stained with Sybr Gold. The absence of genomic DNA was initially checked in a real-time PCR containing 50 ng of total RNA (without reverse transcription) and α-tubulin-specific primers (CsTua1). The absence of inhibitors in 250 ng of RNA samples was also checked by SPUD assay with Sybr green [15].

First strand cDNA synthesis was carried out in 20 μL reactions containing 1 μg RNA with RNase H^+ MMLV reverse transcriptase iscript and mix of oligo(dT) and random primers from iscript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The reverse transcription was made up for 5 min at 25 °C, then 30 min at 42 °C and finalized by 5 min at 85 °C. The quality of cDNAs was verified by 5′–3′ ratio (performing real-time PCR with α-tubulin-specific primers:CsTua 4 in 5′-strand and CsTua 6 in 3′-strand; Table 3).

Real-time RT-PCR was performed on 50 ng cDNA using iQ Sybr green supermix containing hot-start iTaq DNA polymerase in an iCycler iQ (Bio-Rad Laboratories, Hercules, CA). Primer pairs used to amplify cucumber plasma membrane H^+-ATPase genes (CsHa1, CsHa2), Fe(III) chelate-reductase gene (CsFRO1), Fe(II) transporter gene (CsIRT1) and α-tubulin (reference gene) were designed with Beacon Premier Biosoft Software and synthesized by Sigma-Genosys (UK) (Table 3).

The real-time PCR program consisted of an iTaq DNA polymerase activation at 95 °C for 3 min, followed by 40 amplification cycles (denaturing step for 10 s at 95 °C; annealing step for 10 s at 62 °C, and elongation step for 10 s at 72 °C during which the fluorescence data were collected). To confirm PCR products, a melting curve was performed by heating the samples from 72 to 98 °C in 0.1 °C increments with a dwell time at each temperature of 10 s during which the fluorescence data were collected. The melting temperature of the products was determined with the iCycler iQ Optical System Software (version 3: Bio-Rad Laboratories).

The reaction efficiency of PCR (comprising between 1.99 and 2.02) was determined on the slope of standard dilution curves of pooled cDNAs with each specific primer pairs at harvest times. Target gene expression was normalised to α-tubulin expression. Relative expression (*n*-fold) of the normalised target gene in the treatment was calculated with the Relative Expression Software Tool-Multiple Condition Solver (REST-MCS-beta software version august 2006), via comparison to control plants considering efficiency values according to the mathematical model proposed by Pfaffl et al. [16].

2.7. Isolation of plasma membrane vesicles and ATPase assays

Plasma membrane (PM) vesicles were isolated from apical roots (3–5 cm) using a sucrose-gradient technique as previously described by Pinton et al. [17] and Santi et al. [18]. Briefly, the root segments corresponding to the different treatments were cut separately and ground with a pestle in an ice-cold homogenization medium containing 250 mM sucrose, 10% (v/v) glycerol, 10 mM

Table 3
Primer sequences used in the gene expression studies.

Sequence definition	GenBank accession number	Product length (bp)	Primer name	Primer sequence	
Cucumis sativus partial mRNA for proton-exporting ATPase (ha1 gene)	AJ703810	303	CsHA1	Sense	5′-GGGATGGGCTGGTGTAGTTTG-3′
				Antisense	5′-GCTATCTCTGCTCGTCTCTTGG-3′
Cucumis sativus partial mRNA for proton-exporting ATPase (ha2 gene).	AJ703811	290	CsHA2	Sense	5′-TGAGCGACCTGGACTTCTATTG-3′
				Antisense	5′-GTGCCCATTGTGCTTCTCTTTC-3′
Cucumis sativus iron-regulated transporter mRNA, complete cds.	AY590764	259	CsIRT1	Sense	5′-TTCGCAGCAGGTATCATTCTCG-3′
				Antisense	5′-CACCACTCACTACAGGCAACTC-3′
Cucumis sativus ferric reductase mRNA, complete cds.	AY590765	152	CsFRO1	Sense	5′-AGCGGCGGCAGTGGAATC-3′
				Antisense	5′-GTTTGGAGGAGGTGGAGGAAGG-3′
Cucumis sativus partial mRNA for alpha-tubulin (tua gene).	AJ715498	129	CsTUA1	Sense	5′-ACCGTTGGAAAGGAAATTGTTG-3′
				Antisense	5′-GGAGCCGAGACCAGAACC-3′
		141	CsTUA4	Sense	5′-ACTACACCGTTGGAAAGGAAATTG-3′
				Antisense	5′-AAAGGAGGGAGCCGAGACC-3′
		149	CsTUA6	Sense	5′-GACATTGAGCGACCTAACTAC-3′
				Antisense	5′-AACTGGATTCTGGGATATGGG-3′

glycerol-1-phosphate, 2 mM $MgSO_4$ 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT (dithiothreitol), 5.7% (w/v) choline–iodine, 1 mM PMFS, 20 μg mL^{-1} chymostatin, and 25 mM BTP (1,3-*bis* [*TRIS*(hydroxymethyl)methylamino]propane) buffered to pH 6.7 with MES. Approximately, 2.5 mL g^{-1} fresh weight of root tissues were used.

The homogenates were filtered through four layers of cheesecloth and subjected to 3 min of centrifugation at 13,000 *g* at 4 °C (Beckman Coulter Microfuge 22R Centrifuge). The pellets were discarded and the suspension was centrifuged for a further 25 min under the same conditions. The pellets were then recovered, gently resuspended in 400 μL homogenization medium, and loaded onto discontinuous density gradients made by layering 700 μL of 25% (w/w) sucrose over 300 μL 38% (v/v) sucrose cushion in 1.5 mL tubes. Both sucrose solutions were prepared in 5 mM BTP-MES, pH 7.4, and contained all the protectants present in the homogenization medium. The gradients were centrifuged for 1 h at 13,000 *g*, and the vesicles banding at the 25/38% interface were collected, diluted and prepared for enzyme activity measurements.

The PM H^+-ATPase activity was determined according to Santi et al. [18] and Forbush [19]. Assays were performed at 38 °C in a 0.6 mL reaction vol. containing 50 mM MES-BTP, pH 6.5, 5 mM $MgSO_4$, 100 mM KNO_3, 600 μM Na_2MoO_4, 1.5 mM NaN_3, 5 mM ATP-BTP, pH 6.5, 0.01% (w/v) Brij 58 (polyoxyethylene 20 cetyl ether), plus or minus 100 μM V_2O_5. The reaction was started with the addition of membrane vesicles (0.5 μg of total protein); after 30 min, the reaction was blocked and colour developed as described in Veranini et al. [20]. Inorganic phosphate concentration was determined spectrophotometrically at 705 nm, and PM H^+-ATPase activity was expressed as that inhibited by 100 μM vanadate.

2.8. Ferric-chelate reduction by intact roots

Fe(III)–EDTA reduction by roots of cucumber plants was measured as reported by Pinton et al. [4] using the bathophenanthrolinedisulfonate (BPDS) reagent. Briefly, 1 g of the root of a single plant were incubated in 5 mL of the nutrient solution containing Fe(III)–EDTA 0.387 mM, and BPDS 0.286 mM (pH 5.5) in darkness at 25 °C. After 30 min, the absorbance of the solution was measured at 535 nm and the quantity of Fe(III) reduced was calculated by the concentration of the Fe(II)–BPDS complex formed, using an extinction coefficient of 22.1 mM^{-1} cm^{-1}. We observed in previous experiments a good reproducibility of the results obtained using this method, as well as a good correlation between these results and those obtained using the whole root of the plant (data not shown).

2.9. Protein assay

Protein concentrations were determined following Bradford technique using BSA as standard [21].

2.10. Statistical analysis

Significant differences ($p < 0.05$) of enzymatic activities among treatments were calculated by using one-way analysis of variance (ANOVA) and the Tuckey-*b* post hoc test. Statistical analysis of relative expression results was assessed using Pair Wise Fixed Reallocation Randomisation Test.

3. Results

3.1. The PHA characterization

The chemical composition of PHA was 59.40% C, 1.30% N, 2.40% H, and 36.8% O, whereas that of IHSS LHA was 62.20% C, 1.20% N, 3.60% H, and 30.50% O.

The acidic functional group concentration, obtained using potentiometric analysis, was 1.98 mmol g^{-1} of carboxylic groups and 1.18 mmol g^{-1} of phenolic groups for PHA; and 1.12 mmol g^{-1} of carboxylic groups and 0.83 mmol g^{-1} of phenolic groups for IHSS LHA.

Regarding the functional C distribution, ^{13}C NMR studies indicated that PHA contained 27.4% of alkyl C, 12.6% of *O*-alkyl C, 46.5% of aromatic C, 12.8% of phenolic C, 12.2% of carboxylic C and 1.3% of carbonylic C (Fig. 1). In the case of IHSS LHA the functional C distribution was 26.6% of alkyl C, 14.9% of *O*-alkyl C, 48.7% of aromatic C, 12.8% of phenolic C, 9% of carboxylic C and 0.8% of carbonylic C (Fig. 1).

Finally, the PHA size distribution obtained by HPSEC showed a main peak with a maximum corresponding to an MW of 61,200 Da (elution volume: 7.50 mL) (Fig. 2). This peak included an MW interval of 4250–461,000 Da (elution volume: 6.45–9.60 mL). In the case of IHSS LHA, the main peak corresponded to an MW of 68,500 Da (elution volume: 7.43 mL). This peak included an MW interval of 2850–638,000 Da (elution volume: 6.30–9.93 mL) (Fig. 2).

As for the analysis of the main plant hormones in PHA, the results obtained showed that the concentration of the different plant regulators studied was below detection limits.

3.2. Iron concentration in plant tissues and plant growth

The effects of different doses of PHA on the Fe concentration in both root and shoot of PHA-treated and control plants are presented in Fig. 3. No significant differences were observed among

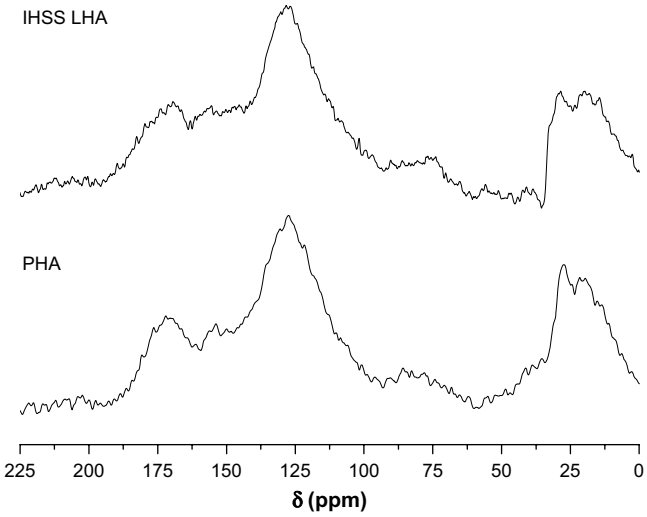

Fig. 1. ^{13}C CPMAS NMR spectra of the standard humic acid (IHSS LHA) and the purified humic acid (PHA).

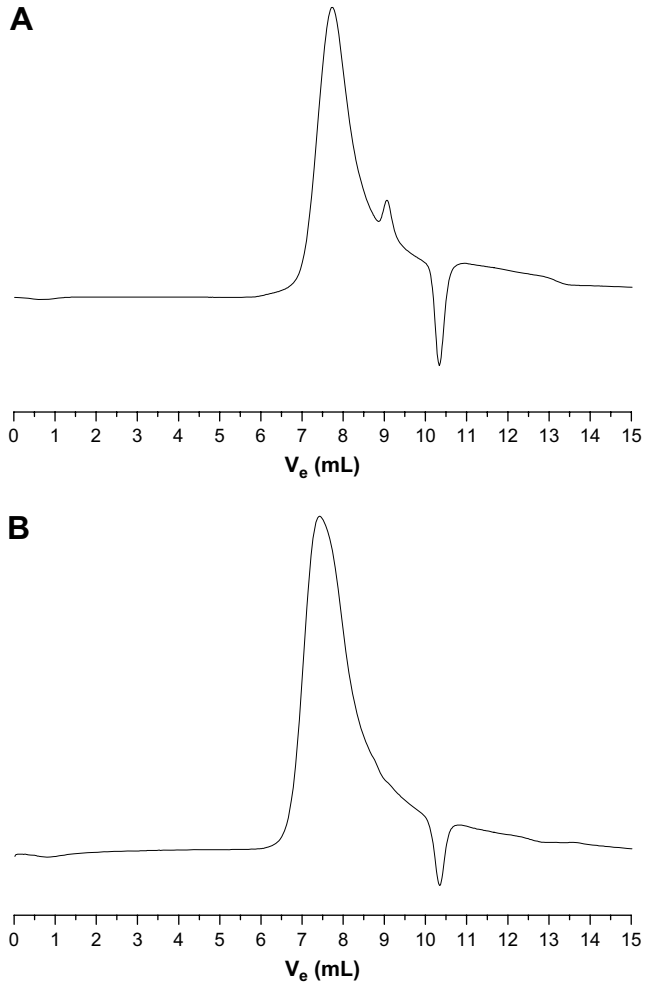

Fig. 2. HPSEC chromatograms obtained with the refractive index detector showing the molecular size distribution for the purified humic acid (PHA) (A) and the standard humic acid (IHSS LHA) (B).

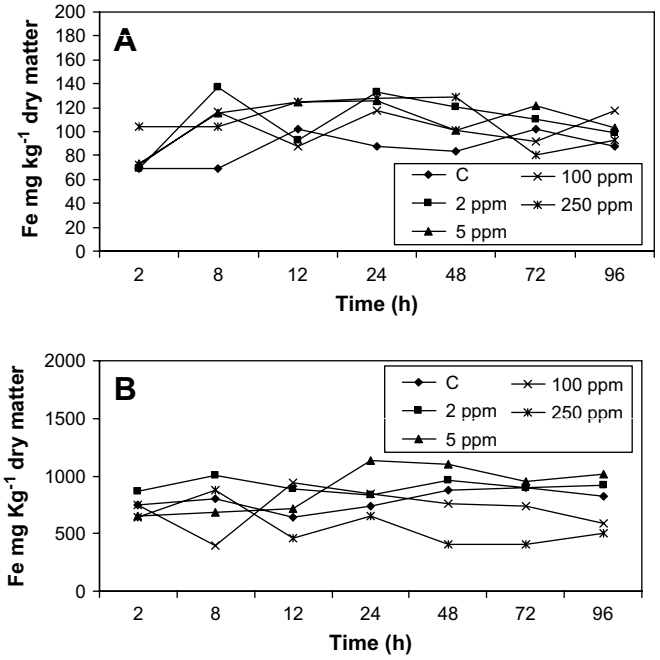

Fig. 3. Time course of the concentration of Fe in both leaves (A) and roots (B) of PHA-treated and control plants (values are the means of three replicates). No significant differences at $p < 0.05$ among treatments were observed.

the different PHA treatments and the control. Regarding plant growth, there were no significant differences among the different PHA treatments and the control in the time period of the study (96 h) (data not shown).

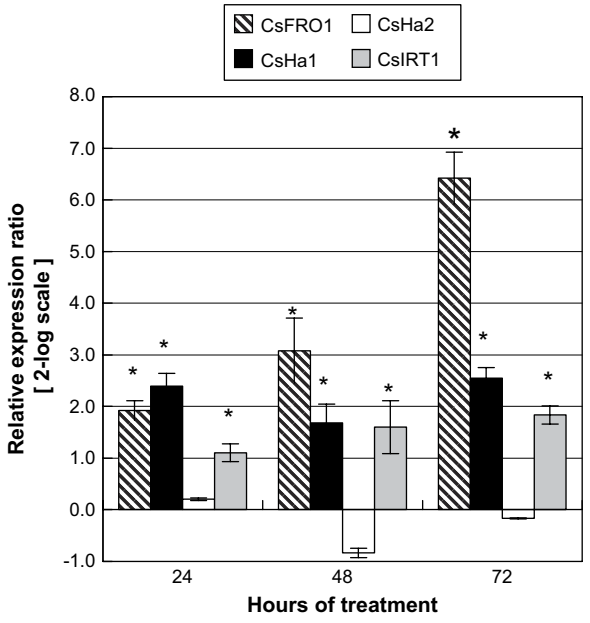

Fig. 4. Time course of the transcriptional expression of the plasma membrane H^+-ATPase genes (CsHa1 and CsHa2), the Fe(III) chelate-reductase (CsFRO1 gene), and the Fe(II) root transporter (CsIRT1 gene) in apical roots of plants subjected to Fe deficiency. Quantitative real-time RT-PCR was performed on root apices of cucumber harvested at different times from the onset of the treatments and excised for isolation of mRNA, as detailed in Section 2. The values are the means of three replicates, run in triplicate. Expression ratio was calculated on the basis of the expression level of studied gene in control plants. mRNA levels were normalised with respect to the internal control α-tubulin. Error bars represent REST error calculation using Taylor's series and statistical significance at 0.05 level ($p < 0.05$) by Randomisation Test is indicated by the asterisk above the column.

3.3. Transcriptional expression

As shown in Fig. 4, the principal molecular agents involved in iron root assimilation presented a greatly enhanced expression in response to Fe deficiency. These results confirm the suitability of the used primers to study the mRNA accumulation of studied genes: CsHA1 and CsHA2, CsFRO1 and CsIRT1 [22,23].

Concerning the effect of PHA on the expression of the above-mentioned gene, our results indicated that CsHas are differentially expressed in the presence of PHA. Indeed, real-time PCR quantitation showed that PHA down-regulated CsHa1 isogene, principally in response to the highest PHA dose (up to 5-fold) (Fig. 5A) whereas CsHa2 transcripts were enhanced after 24 h of PHA treatment (Fig. 5B). This positive effect was higher for all PHA doses after 48 h from the onset of treatment, with the dose 100 mg C L^{-1} showing the maximum effect (4-fold over the control) (Fig. 5B).

The PHA treatment also up-regulated the expression of CsFRO1 and CsIRT1 gene after 48 and 72 h from the onset of the treatments (Fig. 5C and D). Thus, when compared with control roots, the Fe(III) chelate-reductase transcript level in PHA-treated roots was greatly increased, between 2- and 4-fold, by the action of all PHA doses after 48 h from the onset of the treatment. The maximum effect (up to 9-fold) was observed after 72 h of treatment with the higher doses of PHA (Fig. 5C). Transcripts of CsIRT1 were also enhanced up to 7-fold over the control by PHA treatment after 48 h (for all PHA doses) (Fig. 5D). This positive effect was maintained till 72 h after treatment for 100 and 250 mg C L^{-1} treated roots. However, these dose of PHA down-regulated both genes for 96 h.

3.4. Enzyme activity

The different dose of PHA significantly tended to stimulate PM H^+-ATPase hydrolytic activity in membrane vesicles isolated from roots after 4, 48 and 96 h from the onset of the treatments (Fig. 6A).

A similar stimulant effect of PHA on Fe(III) chelate-reductase activity was observed after 72 h from the onset of the treatments (Fig. 6B).

4. Discussion

A number of studies have reported the possible relationships between the effects of HS on plant growth and their capacity to improve Fe plant uptake under Fe-deficient conditions [1,2]. In fact, Pinton et al. [4] demonstrated that specific fractions of HS could enhance some of the plant responses to Fe deficiency, such as PM H^+-ATPase and Fe(III) chelate-reductase root activities. However, the question that arises is if this effect of HS on plant Fe uptake mechanisms is only expressed under conditions of Fe deficiency, when Fe deficiency has triggered these mechanisms as a kind of synergic effect favouring the intensity of plant responses under Fe starvation; or whether this effect of HS is also expressed in Fe-sufficient plants. Nevertheless, if the latter case is true, the mechanism of action of HS of Fe-stress root responses might be related to that involved in the plant response to Fe deficiency, or to an alternative, but complementary, pathway.

Our study shows that the root application of a purified humic acid obtained from leonardite (PHA) significantly affected the main

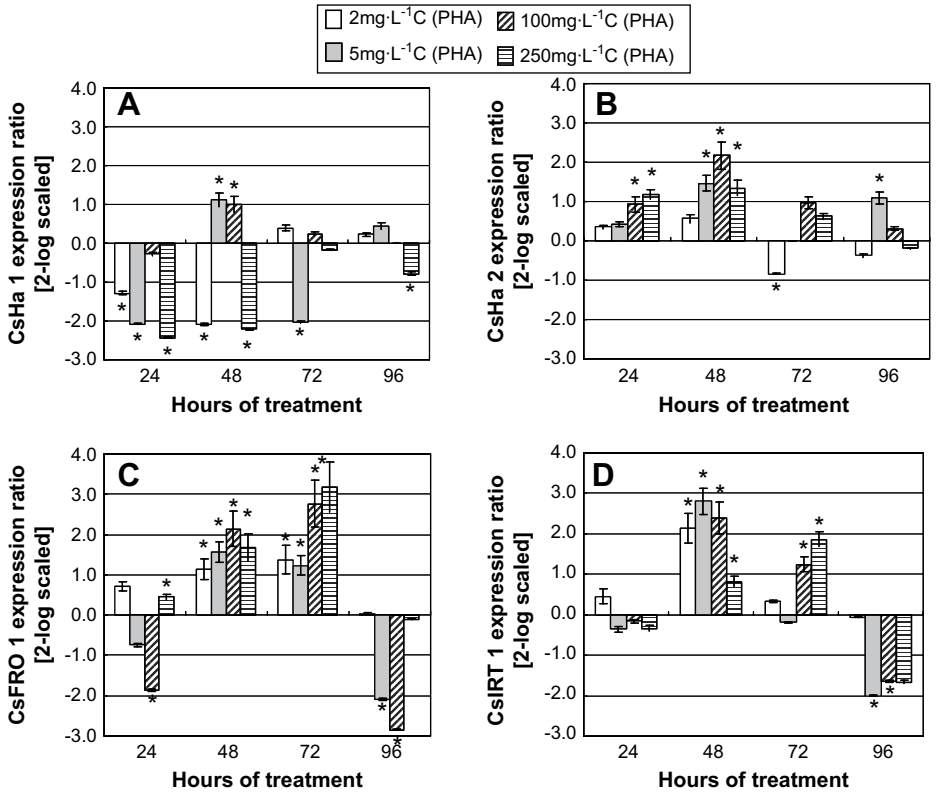

Fig. 5. Time course of the transcriptional expression of the Fe(III) chelate-reductase (CsFRO1), the plasma membrane H^+-ATPase genes (CsHa1 and CsHa2) and the Fe(II) root transporter (CsIRT1) in apical roots exposed to different doses of PHA. Quantitative real-time RT-PCR was performed on root apices of cucumber harvested at different times from the onset of the treatments and excised for isolation of mRNA, as detailed in Section 2. The values are the means of three replicates, run in triplicate. Expression ratio was calculated on the basis of the expression level of studied gene in control plants. mRNA levels were normalised with respect to the internal control α-tubulin. Error bars represent REST error calculation using Taylor's series and statistical significance at 0.05 level ($p < 0.05$) by Randomisation Test is indicated by the asterisk above the column.

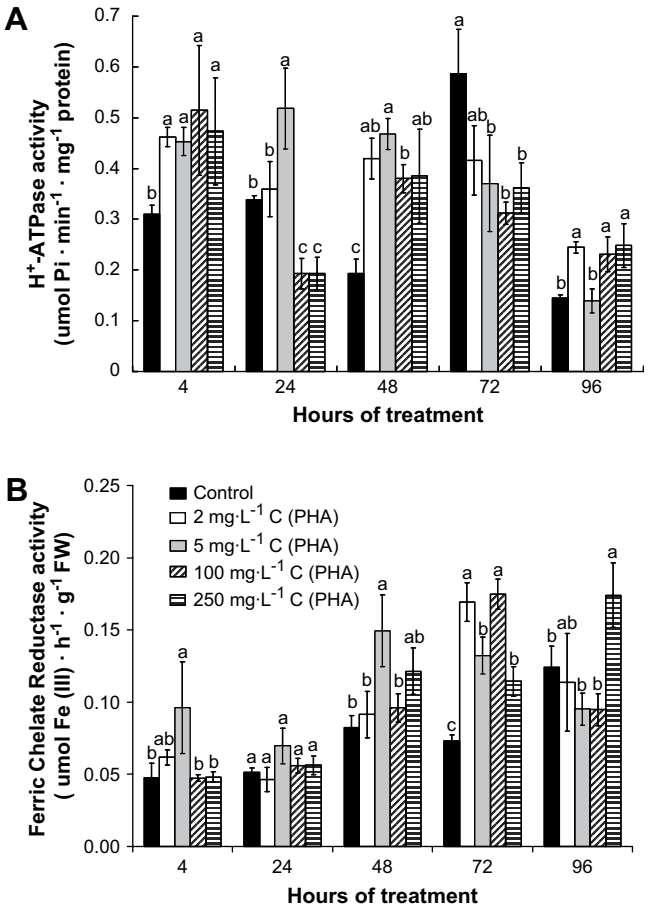

Fig. 6. Effect of different doses of PHA on the plasma membrane H^+-ATPase activity (A), and the Ferric chelate-reductase activity (B) at the indicated times from the onset of the treatments. Plasma membranes vesicles were isolated from the root apices of cucumber. The inhibitor-sensitive activity was calculated by subtracting the ATP-hydrolytic activity from the activity in the presence of vanadate. Ferric chelate reduction was measured in cucumber intact roots as described in materials and methods. The values are the means ± standard error of three replicates. Different letters above the column indicate that the values are significantly different at 0.05 level ($p < 0.05$) according to Tuckey-*b* test.

physiological plant responses in strategy I plants to Fe deficiency, in Fe-sufficient cucumber plants. However, the magnitude and nature (stimulation or inhibition) of these effects were different depending on the gene studied.

Thus, the higher doses of PHA significantly affected the mRNA accumulation of both CsHa isogenes encoding for the PM H^+-ATPase in the roots of Fe-sufficient cucumber plants (Fig. 5A and B). The results showed that the higher doses of PHA caused a transient increase in the expression of CsHa2 isogene that was very significant 48 h from the onset of PHA treatments. This effect was accompanied by a down-regulation of CsHa1 isogene expression with respect to transcript abundance in non-treated roots. Previously, evidence has been found for the regulation of specific PM H^+-ATPase genes in response to various environmental stimuli, such as the presence of nitrate in the nutrient solution [18], auxin action [24], and iron deficiency [22]. However, in all these cases the Ha isoform up-regulated was principally the Ha1. In the case of the treatment with PHA, however, the up-regulated isoform was the Ha2. This fact suggests that in the case of PM H^+-ATPase, regulatory pathways different from and complementary to those involved in Fe deficiency plant responses might mediate the effect of PHA. These results support the idea that the different isoforms may function in different metabolic states and under different environmental conditions, thus leading to enzymes individually tailored either to the cell in which they are expressed or to the function in which they are involved [25]. A similar result was obtained by Quaggiotti et al. [26] in maize. These authors observed that the treatment of maize seedlings with an HS fraction with low molecular weight caused a significant increase in the gene expression of the MHa2 isoform (one of two maize PM H^+-ATPase isoforms: MHa1 and MHa2) after 48 h from the onset of treatments.

Our results showed that PHA effects at transcriptional level on PM H^+-ATPase were also associated with significant increases in the activity of this enzyme for some harvest times. Thus, the increase in the gene expression of CsHa2 for 48 h was associated with a concomitant increase in the PM H^+-ATPase activity (Fig. 6A). This effect at 48 h was preceded by an increase at 4 h. However, the significant increase in the CsHa2 expression at 24 h for the higher PHA doses (100 and 250 mg organic carbon L^{-1}) was not associated with an increase in enzyme activity. This discrepancy of stimulations at transcriptional and post-transcriptional levels could reflect the existence of regulatory mechanisms at both levels, as previously reported for PM H^+-ATPase in cucumber under different experimental conditions [27]. Other authors have also observed the capacity of HS of different origin to increase PM H^+-ATPase activity in the roots of diverse plant species [4,17,20,28–31]. As in our case, some of these studies showed that this effect was transient but significant [17]. In some cases, these effects on H^+-ATPase activity were correlated with increases in nutrient root uptake (principally nitrate) [17], the development of specific morphological changes in the root [28,30], and the effects on root growth [31].

Our results also show that the expression of the genes encoding the Fe(III) chelate-reductase (CsFRO1) and an Fe(II) root transporter (CsIRT1) was also affected by PHA treatments (Fig. 5C and D). As in the case of the effect on CsHa2 gene, these effects were transient and especially significant for 48 and 72 h from the onset of treatments (Fig. 5C and D). It is interesting to note that this up-regulation was followed by a significant down-regulation at 96 h for both genes. This fact may be related to a feedback inhibitory mechanism associated with some event (perhaps root iron accumulation and/or translocation) linked to the previous activation. In the case of the Fe(III) chelate-reductase, the increase in CsFRO1 expression was associated with an increase in the enzyme activity for 72 h (Fig. 6B). These effects were not accompanied by a reduction of the Fe concentration both in the roots and leaves (Fig. 3). Other studies have reported the capacity of humic compounds to affect Fe(III) chelate-reductase activity in Fe-deficient cucumber plants [4]. However, in principle our results show the capacity of a model HS to up-regulate some of the main molecular mechanisms (the Fe(III) chelate-reductase/Fe(II) transporter system) involved in Fe root uptake in the absence of a previous stimuli linked to Fe deficiency. However, the fact that these results could be associated with an effect of PHA on Fe root assimilation and further Fe root–shoot translocation cannot be completely ruled out. Likewise, our experiments do not permit us to elucidate if this effect of PHA on the Fe(III) chelate-reductase/Fe(II) transporter system is mediated by a mechanism similar to that activated by Fe deficiency or by another, alternative, one. In this sense, the fact that PHA affected an H^+-ATPase gene isoform (Ha2) different from that activated under Fe deficiency suggests that the mechanism of action of PHA on the Fe(III) chelate-reductase/Fe(II) transporter system in cucumber may be different from that involved under Fe deficiency.

The absence of clear dose–effect relationships could be due to the variation in the conformational and molecular pattern of the humic system in solution associated with humic concentration [12]. Thus, low PHA concentrations are related to more open and less aggregated structures, whereas higher concentrations favour molecular aggregation and the blocking of biologically active functional sites.

It is interesting to note that these effects at transcriptional and enzyme activity level were associated with the presence of a humic acid with physicochemical characteristics within the range of an IHSS standard leonardite humic acid (Figs. 1 and 2). Likewise, this effect of PHA affecting root Fe uptake mechanisms is ascribed to PHA structure and not to the presence of plant regulators in the PHA structure, since the concentration of the main plant regulators in PHA was neither significant nor measurable.

In any case, these results stress the important relationships existing between HS effects on plant growth and plant Fe uptake mechanisms, as suggested by Chen et al. [1,2]. Regarding the mechanism of action at biochemical and physiological level, some authors suggest that the action of HS on plant growth might also be related to the presence of molecules with auxin activity [28,32,33]. However, these same authors indicated that the concentration of IAA or IAA analogues normally found in HS is too low to explain the effect of HS on plant growth [32]. In fact, as in our case, other authors did not find any traces of phyto-hormones in HS extracted from different organic materials [2]. In this sense, recent studies in *Arabidopsis* using specific mutants indicated that the mechanism of action responsible for the biological activity of HS might be different from that involved in auxin and ethylene action [34]. Further work is needed, therefore, in order to clarify whether these effects of HS at molecular level are founded on mechanisms with nutritional and/or phytoregulatory character.

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References

[1] Y. Chen, C.E. Clapp, H. Magen, Mechanism of plant growth stimulation by humic substances: the role of organo-iron complexes, Soil Sci. Plant Nutr. 50 (2004) 1089–1095.
[2] Y. Chen, M. De Nobili, T. Aviad, Stimulatory effects of humic substances on plant growth, in: F. Magdoff, R.R. Weil (Eds.), Soil Organic Matter in Sustainable Agriculture, CRC Press., Boca Raton, Florida, 2004, pp. 103–129.
[3] J.M. Garcia-Mina, M.C. Antolín, M. Sánchez-Diaz, Metal-humic complexes and plant micronutrient uptake: a study based on different plant species cultivated in diverse soil types, Plant Soil 258 (2004) 57–68.
[4] R. Pinton, S. Cesco, S. Santi, F. Agnolon, Z. Varanini, Water-extractable humic substances enhance iron deficiency responses by Fe-deficient cucumber plants, Plant Soil 210 (1999) 145–157.
[5] V. Römheld, H. Marschner, Different strategies in higher plants in mobilization and uptake of iron, J. Plant Nutr. 9 (1986) 695–713.
[6] H.F. Bienfait, Mechanisms in Fe-efficiency reactions of higher plants, J. Plant Nutr. 11 (1988) 605–629.
[7] C. Curie, J.F. Briat, Iron transport and signalling in plants, Ann. Rev. Plant Biol. 24 (2003) 183–216.
[8] R. Hell, U.W. Stephan, Iron uptake, trafficking and homeostasis in plants, Planta 216 (2003) 541–551.
[9] W. Schmidt, Iron stress responses in roots of strategy I plants, in: L.L. Barton, J. Abadía (Eds.), Iron Nutrition in Plants and Rhizospheric Microorganisms, Springer, Dordrecht, 2006, pp. 229–250.
[10] J.M. García-Mina, Stability, solubility and maximum metal binding capacity in metal–humic complexes involving humic substances extracted from peat and organic compost, Org. Geochem. 37 (2006) 1960–1972.
[11] T. Takamatsu, T. Yoshida, Determination of stability constants of metal–humic acid complexes by potentiometric titration and ion-selective electrodes, Soil Sci. 125 (1978) 377–386.
[12] R. Baigorri, M. Fuentes, J.M. García-Mina, G. González-Gaitano, Analysis of molecular aggregation in humic substances in solution, Colloids Surf. A 302 (2007) 301–306.
[13] P.I. Dobrev, M. Kamínek, Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction, J. Chromatogr. A 950 (2002) 21–29.
[14] F.J. Romera, E. Alcantara, M. Díaz de la Guardia, Ethylene production by Fe-deficient roots and its involvement in the regulation of Fe-deficiency stress responses by strategy I plants, Ann. Bot. 83 (1999) 51–55.
[15] T. Nolan, R.E. Hands, W. Ogunkolade, S.A. Bustin, SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations, Anal. Biochem. 351 (2006) 308–310.
[16] M.W. Pfaffl, G.W. Horgan, L. Dempfle, Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real time PCR, Nucleic Acids Res. 30 (2002) e36.
[17] R. Pinton, S. Cesco, G. Iacolettig, S. Astolfi, Z. Varinini, Modulation of NO_3^- uptake by water-extractable humic substances: involvement of root plasma membrane H^+ ATPase, Plant Soil 215 (1999) 155–161.
[18] S. Santi, G. Locci, R. Monte, R. Pinton, Z. Varanini, Induction of nitrate uptake in maize roots: expression of a putative high-affinity nitrate transporter and plasma membrane H^+-ATPase isoforms, J. Exp. Bot. 54 (2003) 1851–1864.
[19] B. Forbush, Assay of the Na^+ K^+-ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using dodecyl sulfate buffered with bovine serum albumin, Anal. Biochem. 128 (1983) 159–163.
[20] Z. Veranini, R. Pinton, M.G. De Biasi, S. Astolfi, A. Maggioni, Low molecular weight humic substances stimulate H^+-ATPase activity of plama membrane vesicles isolated from oat (*Avena sativa* L.) roots, Plant Soil 153 (1993) 61–69.
[21] M.M. Bradford, A rapid and sensitive method of quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[22] C. Santi, S. Cesco, Z. Varanini, R. Pinton, Two plasma membrane H^+-ATPase genes are differentially expressed in iron-deficient cucumber plants, Plant Physiol. Biochem. 43 (2005) 284–292.
[23] B.M. Waters, C. Lucena, F.J. Romera, G.G. Jester, A.N. Wynn, C.L. Rojas, E. Alcantara, R. Perez-Vicente, Ethylene involvement in the regulation of the H^+-ATPase CsHA1 gene and of the new isolated ferric reductase CsFRO1 and iron transporter CsIRT1 genes in cucumber plants, Plant Physiol. Biochem. 45 (2007) 293–301.
[24] I. Frías, M.T. Caldeira, J.R. Pérez-Castiñeira, J.P. Navarro-Aviño, F.A. Culiañez-Maciá, O. Kuppinger, H. Stransky, M. Pagés, A. Hager, R. Serrano, A major form of the maize plasma membrane H^+-ATPase: characterization and induction by auxin in coleoptiles, Plant Cell 8 (1996) 1533–1544.
[25] M. Arango, F. Gévaudant, M. Oufattole, M. Boutry, The plasma membrane proton pump ATPase: the significance of gene subfamilies, Planta 216 (2003) 355–365.
[26] S. Quaggiotti, B. Ruperti, D. Pizzeghello, O. Francioso, V. Tugnoli, S. Nardo, Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.), J. Exp. Bot. 55 (2004) 803–813.
[27] E. Bacaicoa, E. Aguirre, D. Lemenager, A.M. Zamarreño, R. Baigorri, J.M. Garcia-Mina, Hormonal balance in iron-efficient cucumber plants grown under sufficient and deficient conditions of iron availability, in: 13th International Symposium on Iron Nutrition and interactions in plants, Agro-M/INRA Montpellier, 2006, p. 87.
[28] L.P. Canellas, F.L. Olivares, A.L. Okorokova-Façanha, A.R. Façanha, Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+-ATPase activity in maize roots, Plant Physiol. 130 (2002) 1951–1957.
[29] R. Pinton, S. Cesco, W. Schmidt, Z. Varanini, Role of humic substances as rhizospheric signals affecting root growth and mechanisms of nutrient acquisition, in: F.H. Frimmel, G. Abbt-Braun (Eds.), Humic Substances-linking Structure to Functions, Vol. 45 (I), Schriftenreihe Bereich Wasserchemie/Engler-Bunte-Institut der Universität Karlsruhe, 2006, pp. 45–48.
[30] D. Zandonadi, L.P. Canellas, A.R. Façanha, Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H^+ pumps activation, Planta 225 (2007) 1583–1595.
[31] L. Canellas, D.B. Zandonadi, J.G. Busato, M.A. Baldoto, M.L. Simoes, L. Martin-Neto, A.R. Façanca, R. Spaccini, A. Piccolo, Bioactivity and chemical characteristics of humic acids from tropical soil sequence, Soil Sci. 173 (2008) 624–637.
[32] S. Nardi, D. Pizzeghello, A. Muscolo, A. Vianello, Physiological effects of humic substances on higher plants, Soil Biol. Biochem. 34 (2002) 1527–1536.
[33] A. Muscolo, S. Cutrupi, S. Nardi, IAA detection in humic substances, Soil Biol. Biochem. 30 (1998) 1199–1201.
[34] W. Schmidt, S. Santi, R. Pinton, Z. Varanini, Water-extractable humic substances alter root development and epidermal cell pattern in *Arabidopsis*, Plant Soil 300 (2007) 259–267.