

Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H⁺ pumps activation

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Abstract Increasing evidences have indicated that humic substances can induce plant growth and productivity by functioning as an environmental source of auxinic activity. Here we comparatively evaluate the effects of indole-3-acetic acid (IAA) and humic acids (HA) isolated from two different soils (Inseptisol and Ultisol) and two different organic residues (vermicompost and sewage sludge) on root development and on activities of plasmalemma and tonoplast H⁺ pumps from maize roots. The data show that HA isolated from these different sources as well as low IAA concentrations (10⁻¹⁰ and 10⁻¹⁵ M) improve root growth through a markedly proliferation of lateral roots along with a differential activation not only of the plasmalemma but also of vacuolar H⁺-ATPases and H⁺-pyrophosphatase. Further, the vacuolar H⁺-ATPase had a peak of stimulation in a range from 10⁻⁸ to 10⁻¹⁰ M IAA, whereas the H⁺-pyrophosphatase was sensitive to a much broader range of IAA concentrations from 10⁻³ to 10⁻¹⁵ M. It is proposed a complementary view of the acid growth mechanism in which a concerted activation of the plasmalemma and tonoplast H⁺

pumps plays a key role in the root cell expansion process driven by environment-derived molecules endowed with auxinic activity, such as that of humic substances.

Keywords Cell expansion · H⁺-PPase · Humic substances · Lateral root initiation · Plant growth regulators · V-ATPase

Abbreviations

HA	Humic acids
H ⁺ -PPase	Membrane-bound pyrophosphatase
IAA	Indole-3-acetic acid
P-ATPase	Plasma membrane H ⁺ -adenosine triphosphatase
V-ATPase	Vacuolar H ⁺ -adenosine triphosphatase
V-PPase	Vacuolar H ⁺ -pyrophosphatase

Introduction

Plants sense and respond to endogenous and environmental signals to ensure optimal growth and development using phytohormones as chemical messengers (Swarup et al. 2002). Auxins are hormones involved in plant-cell elongation, apical dominance and rooting. A well-known natural auxin is indole-3-acetic acid (IAA), which is endogenously produced in the apical meristem of the shoot and can be rapidly transported to the roots via the phloem. Lateral roots originate from mature, nondividing pericycle cells within the parent root. Auxinic signals trigger groups of pericycle cells to re-enter the cell cycle and establish lateral root mitotic sites (Casimiro et al. 2001; Bhalerao et al. 2002). These cells undergo a well-defined program of

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oriented cell divisions and produce a patterned lateral root primordium containing all the differentiated cell types of a mature root (Charleton 1991). This does not necessarily imply that root morphology is only under the control of endogenous auxins. Indeed, the experimentally observed lateral root induction by exogenous IAA could be seen as a normal phenomenon since auxins are commonly present in the natural environments. It has long been recognised that soils, particularly those rich in decaying organic matter, contain auxins (Whitehead 1963) and humic substances that can express auxinic and other hormonal activities on plant development (e.g., Bottomley 1917; Guminsk 1968; Mato et al. 1972; Cacco and Dell'Agnola 1984; Nardi et al. 2002; Canellas et al. 2002; Quaggiotti et al. 2004). However, although there has been an increasing interest in the signalling of endogenous auxin on root development, very little work has been done in analysing the mode of action of exogenous environmentally derived auxins.

Humic substances are the major components of soil organic matter, and they have a prominent importance in sustaining life on earth (Canellas et al. 2005). It is now well-established that humic substances improve the plant development by influencing, directly or indirectly, several plant biochemical processes rather than functioning only as a potential source of nutrients (Vaughan and Malcolm 1985; Nardi et al. 2002, and references therein). Despite such an important role, the basic chemical nature of humic substances and their mechanisms of action remain poorly understood. Humic acids (HA) comprise one of the major fractions of humic substances and for a long time their basic structural unit was described just as a single molecule of high molecular weight originated by microbial biopolymers transformation in soils and another sources of organic matter (Kononova 1966; Schnitzer 1991). However, recent information gathered using chromatographic and NMR techniques has improved this understanding of HA structure by describing it as supramolecular associations of relatively smaller organic molecules clustered basically by hydrophobic interactions and hydrogen bonds (Piccolo 2001; Simpson 2002). Based on such a concept, we have explored the biochemical properties of bioactive molecules forming the supramolecular structure of HA related to their hormone-like effects on root development (Canellas et al. 2005, and references therein). We have verified the presence of indoleacetic groups in HA structure of vermicompost, by means of gas chromatography-mass spectrometry. From these studies, a mechanism for HA bioactivity based on the classical acid growth theory has been proposed, describing an

auxin-like induction of protein synthesis and activation of the plasma membrane H^+ -ATPase in maize roots (Canellas et al. 2002; Façanha et al. 2002). These findings were reinforced and extended by Quaggiotti et al. (2004), which have also detected IAA, by immunoassays, in low molecular size humic fractions also isolated from vermicompost, and demonstrated the induction of the *Mha2* gene, which encode one of the main P-type H^+ -ATPase isoforms expressed in maize root cells.

However, during plant cell growth cycle, following meristematic cell enlargement, vacuolation (turgor maintenance capacity of vacuole) provides volume expansion necessary for the cell elongation (Cosgrove 2000). Therefore, the tonoplast H^+ pumps, a V-type H^+ -ATPase and a membrane-bound pyrophosphatase (H^+ -PPase), could also play a role in the cell growth process, since the H^+ electrochemical gradient generated by these pumps energize secondary transporters that maintain the osmotic pressure of the vacuole sufficiently high for water uptake and vacuolation (Mae-shima et al. 1996; Smart et al. 1998). Despite this, the relative importance of the two vacuolar H^+ pumps in root cell expansion is still unclear (Dolan and Davies 2004).

Hormonal effects on plant development have widely been described for HA originated from different sources, like soils (e.g., Govindasmy and Chandrasekaran 1992; Pizzeghello et al. 2001), organic residues as sludge (Masciandaro et al. 1999; Façanha et al. 2002) and earthworm composts (Cacco and Dell'Agnola 1984; Masciandaro et al. 1999; Atiyeh et al. 2002; Canellas et al. 2002). In this study, we have analysed HA isolated from two different soils (Insepsol and Ultisol) and two different organic residues (vermicompost and sewage sludge) in order to analyse structural and functional aspects between the natural and anthropogenic sources of organic matter. The effects of these HA and different concentrations of IAA on tonoplast H^+ pumps have been investigated as a putative biochemical mechanism by which HA could express their hormonal activities in influencing root growth and morphology.

Materials and methods

Isolation and chemical characterisation of HA

Humic acids were isolated from earthworm compost (vermicompost) of cattle manure (HAV), sewage sludge samples collected at different sites in a treatment plant of Rio de Janeiro city (HAS), and topsoil

horizons from Ultisol (HAU) and Inceptisol of Campos dos Goytacazes, Rio de Janeiro, Brazil. The humic substances were extracted as described by the International Humic Substance Society (Schnitzer and Skinner 1982). Elemental composition was determined with a CHN Perkin-Elmer auto-analyser. Ash contents were obtained by incineration of samples at 700°C. Total acidity of HA was estimated by the Ba(OH)₂ method and carboxylic acidity by the Ca(OAc)₂ method according to Schnitzer and Gupta (1965). The humification degree of HA was estimated through the E₄/E₆ ratios as described by Kononova (1982), measuring the absorbances of 20 mg C l⁻¹ HA 0.05 mol pH 8.2 NaHCO₃ samples at 465 and 665 nm in a Hitachi U-2000 spectrophotometer. Fourier transformed infrared (FT-IR) spectra of HA were recorded on KBr pellets in the 400–4,000 cm⁻¹ wave number using a Shimadzu 83000 spectrophotometer. A mixture of 1 mg HA and 300 mg KBr was pressed, under reduced pressure, to obtain the KBr pellets. The signal spectra of H₂O and CO₂ contamination (measured at 3,300–3,400 and 2,340 ± 10 cm⁻¹, respectively) were discounted. To compare one spectrum to another, data were corrected using baselines at 4,000, 2,000 and 860 cm⁻¹, considered as zero transmittance.

Plant growth, HA and IAA treatments

Maize seeds (*Zea mays* L., var. UENF 506/6, provided by LMGV, UENF, Rio de Janeiro, Brazil) were cleaned by soaking in 0.5% NaClO for 30 min, followed by rinsing and then soaking in water for 6 h. Afterward, the seeds were sown on wet filter paper and germinated in the dark at 28°C. Four-day-old maize seedlings with roots of about 2 cm were transferred into a solution containing 2 mM CaCl₂ and 0 or 20 mg C l⁻¹ HA extracted from different sources (HAU-Ultisol; HAI-Inceptisol; HAS-sewage sludge; HAV-vermicompost) or IAA (10⁻³, 10⁻⁵, 10⁻⁶, 10⁻⁸, 10⁻¹⁰ and 10⁻¹⁵ M). These particular types of HA were selected since previous assays showed that seedlings treated for 5 days with different concentration of these specific HA exhibited high rates of relative root growth and no phytotoxic effect for around 20 mg C l⁻¹ HA (data not shown). For clarity, only this HA concentration was used in all experiments since previous analyses have revealed a very complex concentration-dependence for the HA activities. This is consistent with the notion that HA functions as organic matrixes, rather than as a single molecule, releasing bioactive molecules in a complex cross-talking with the roots (Canellas et al. 2005). It is worth noting that these HA improved the plant growth in water as well as in com-

plete nutrient solution in agreement with previously reported data (for a review see: Chen and Aviad 1990). A minimal medium (CaCl₂ 2 mM) has been used in this work in order to avoid any interference of nutrient constituents, which could function synergistically along with HA on the plant growth and metabolism.

Measurements of root growth and lateral root development

On the 5th day of treatment, roots were collected to estimate their surface area using *DeltaTscan*TM (Dynamax Inc.) software image analyser. Whole root systems of treated seedlings were harvested to evaluate the number of mitotic sites and lateral roots emerged as described in Canellas and co-workers (2002). In brief, roots were washed in water and cleared by boiling at 75°C for 20 min in KOH (0.5%, w/v). Afterward, the roots were rinsed in water and stained for 14 h in the dark in haematoxylin staining solution; rinsed once again in water and destained in 80% lactic acid at 75°C for 30–90 s. Individual sample was observed using stereoscopic microscopy at 40× to evaluate the number of mitotic sites, visible as red dots on a pink to white background of root tissue. Root fresh weight was also measured and another sample of root seedlings were collected and used for further experiments.

Plasma membrane and tonoplast-enriched vesicles preparation

Plasma membrane and tonoplast vesicles were isolated from roots grown with and without 20 mg C l⁻¹ HA or IAA using differential centrifugation as described by Bennett and co-workers (1984), with some modifications (Façanha and de Meis 1998). Briefly, about 10 g (fresh weight) of maize roots were homogenized using a mortar and pestle in 20 ml of ice-cold buffer containing 250 mM sucrose, 10% (w/v) glycerol, 2 mM EDTA and 0.1 M Tris-HCl buffer, pH 8.0. Just prior to use, 150 mM KCl, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% (w/v) bovine serum albumin (BSA) and 0.5% (w/v) polyvinylpyrrolidone-40 (PVP-40 kDa) were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at 8,000 g for 10 min. The supernatant was centrifuged once more at 8,000 g for 10 min and then at 100,000 g for 40 min. The pellet was resuspended in a small volume of ice-cold buffer containing 10 mM Tris-HCl (pH 7.6), 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF and 1 mM EDTA. The suspension containing membrane vesicles was layered over a 25/45% (w/w) discontinuous sucrose gradient

that contained, in addition to sucrose, 10 mM Tris–HCl buffer (pH 7.6), 1 mM DTT, 1 mM PMSF and 1 mM EDTA. After centrifugation at 100,000 g for 90 min in a swinging bucket rotor, two bands were found localized at the interface between 25 and 45% layers and on the top of the 25% layer, containing vesicles derived from plasmalemma and tonoplast, respectively. These vesicles were collected and either used immediately or frozen under liquid N₂ and stored at –70°C until use. Protein concentration was determined as described by Bradford (1976).

ATP and pyrophosphate hydrolysis activities

ATPase activity obtained from plasmalemma and tonoplast vesicles was determined by measuring the release of inorganic phosphate colorimetrically (Fiske and Subbarow 1925) at 30°C. The assay medium contained 20 mM Mops–Tris (pH 7.0 for tonoplast vesicles or 6.5 for plasma membranes vesicles), 100 mM KCl, 5 mM MgCl₂ and 0.05 mg l⁻¹ vesicles protein. The V-ATPase activity was measured, with and without 50 mM KNO₃ or 50 nM concanamycin A, two inhibitors of V-ATPases (Sze 1985; Huss et al. 2002), and the difference between the activities with and without the inhibitors was attributed to the tonoplast H⁺-ATPase. The vacuolar K⁺ activated pyrophosphate (PPi) hydrolysis was calculated through the difference of the activity obtained in the presence and that in the absence of KCl. The plasma membrane H⁺-ATPase activity was measured with or without 0.2 mM vanadate, an inhibitor of P-type H⁺-ATPases (Sze 1985), and the difference between these two activities was attributed to the plasma membrane H⁺-ATPase. The reactions were started by addition of 1 mM ATP for both ATPases and 0.1 mM PPi for pyrophosphatase activity.

Plasma membrane and vacuolar proton pumping

Proton-pumping capacity of the plasmalemma and tonoplast H⁺-pumps was estimated from the initial rate of quenching of the fluorescent pH probe 9-amino-6-chloro-2-methoxyacridine (ACMA) (2 μM, 415/485 nm excitation/emission), and expressed in percentage quenching per minute. The assay medium contained 10 mM Mops–Tris (7.0 for vacuolar or pH 6.5 for plasma membrane vesicles), 100 mM KCl, 5 mM MgCl₂, 2 μM ACMA and 0.05 mg l⁻¹ plasma membrane or tonoplast vesicles protein.

The specific activities of the H⁺-pumps were calculated by measuring the H⁺ transport in the presence or in the absence of the inhibitors: 0.2 mM vanadate for P

type H⁺-ATPase and 50 nM concanamycin for V type H⁺-ATPase. H⁺ transport mediated by H⁺-PPase was carried in the presence and in the absence of KCl. The reaction was triggered by addition of either 1 mM ATP (for plasmalemma and tonoplast H⁺-ATPases) or 0.1 mM PPi (for tonoplast H⁺-PPase). The addition of either 3 μM FCCP or 2 μM NH₄Cl abolished the H⁺ gradient formed by either ATP or PPi hydrolysis.

Statistical analysis

The HA bioactivity was analysed by analysis of variance (ANOVA) between groups using Tukey test (*P* < 0.05) for means. It was obtained the Pearson correlation coefficients between some chemical characteristics of the HA (elemental composition, E₄/E₆ ratio, phenolic, carboxylic and total acidities) and some parameters related to their bioactivity (root area, number of mitotic sites, lateral root emergence and H⁺-pumps activities).

Results

Structural features of HA

In order to reveal some chemical properties of HA studied and to correlate them with their bioactivities, the elemental composition and other chemical characteristics of these HA were analysed (Table 1). The element contents of the HA ranged from 32.2 to 55.6% to the carbon (C), from 4.1 to 7.0% to the hydrogen (H) from 35.1 to 61.0% to the oxygen (O) and from 2.7 to 4.9% to the nitrogen (N). The highest C content was found in the HAI and the highest values of O content and acidity were found in HAU. Functional carboxyl and hydroxyl groups have been related to biochemical activity of humic substances (Schnitzer and Poapst 1967; Mato et al. 1972; Nardi et al. 2002).

Humic substances are known to contain conjugated double bond systems, which are responsible for their brown colour. Kononova (1982) established an index of humification degree based on the absorbance ratios at 465 and 665 nm, called E₄/E₆ ratio. Table 1 shows that the E₄/E₆ ratios increased following the sequence HAU < HAI < HAS < HAV (from 3.4 to 6.0). Lower E₄/E₆ ratios indicate relatively higher molecular weight, the presence of structures more condensed and, consequently, higher humification degree (Chen et al. 1977). Here, the anthropogenic HA (HAS and HAV) exhibited a much lower humification degree than that derived from soils (HAU and HAI).

Table 1 Elemental contents, functional acidities and E_4/E_6 ratio of soil humic acids (HA) isolated from Ultisol (HAU), Inceptisol (HAI) sewage sludge (HAS) and vermicompost (HAV)

Treatments	Elemental content (g kg ⁻¹)					Acidities (cmol _c kg ⁻¹)			vE_4/E_6 Ratio
	C	H	N	O	Ash	Total	COOH	PhOH	
HAU	322	41	27	610	20	1,340	1,227	113	3.4
HAI	556	48	45	351	17	333	207	126	3.9
HAS	540	70	49	341	7	594	455	129	4.3
HAV	485	56	32	422	5	666	496	170	6.0

Analyses of FT-IR spectra, based on the inherent vibratory property of the HA atoms, are shown in Fig. 1. Each molecule responds to radiation in a different way, which provides different absorption bands in the FT-IR spectra (Martin-Neto et al. 1996). Thus, some information about the structure of functional groups in the organic matter, as well as the nature of their chemical associations and reactivity can be accessed by FT-IR analyses (Stevenson 1994; Johnston and Aochi 1996). The FT-IR spectra of HA share in common a large absorption band at 3,400 cm⁻¹ due to O–H groups and N–H stretching, symmetrical and anti-symmetrical stretching of C–H aliphatic groups at 2,950 and 2,970 cm⁻¹. The absorption band at 1,720 cm⁻¹ is probably due to C=O in carbonyl groups, and the carbonyl stretching mode of secondary amides such as those occurring in proteins and polypeptides is referred to Amide I band and occurred up to 1,650 cm⁻¹. Secondary amides also exhibit another characteristic band (Amide II) at 1,540 cm⁻¹ assignable as the N–H deformation mode as originally described by Coulthup and co-workers (1964). The ionised carboxyl group occurred between 1,610 and 1,550 cm⁻¹ and between 1,450 and 1,300 cm⁻¹, and corresponds to symmetrical and anti-symmetrical vibrations of COO– structure. Absorption bands at 1,237 cm⁻¹ were attributed to phenol deformations and absorption due to the C–O stretching mode of polysaccharides exhibited bands between 1,094 and 1,007 cm⁻¹. The bands at 914 cm⁻¹ were attributed to C–H aromatic deformation. The main differences between FT-IR spectra from HA were found in the finger print region between 1,480 and 900 cm⁻¹. Since all HA have expressed auxin-like activity, it is likely that their bioactive clusters might be in the conserved region of their structures and not in their finger print regions.

Effects of HA and IAA on root development

The number of mitotic sites was measured in the differentiation zone of roots treated with 20 mg C l⁻¹ HA or 10⁻⁵, 10⁻¹⁰ and 10⁻¹⁵ M IAA. All HA tested and the lowest concentrations of IAA (10⁻¹⁰ and 10⁻¹⁵) enhanced about two times the number of mitotic sites

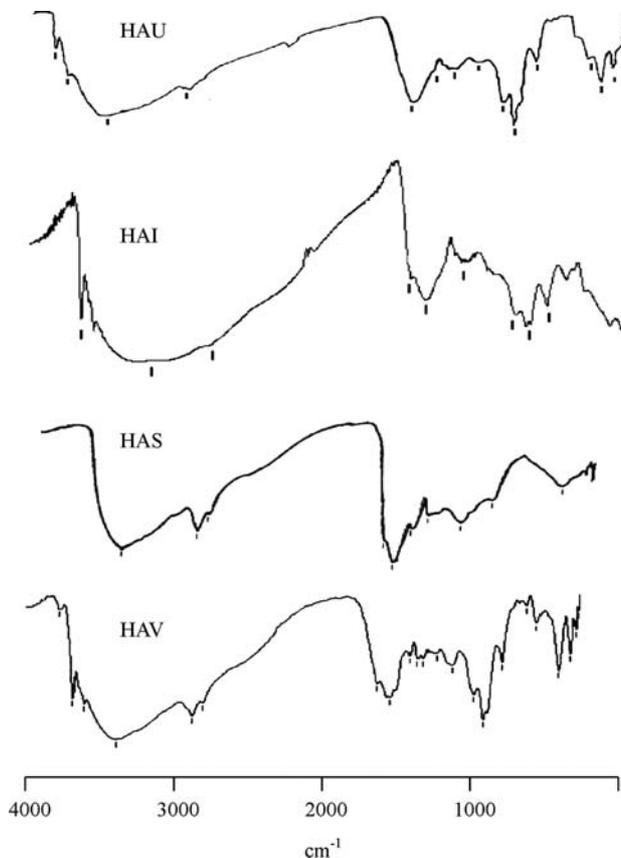


Fig. 1 Fourier-transformed infrared (FT-IR) spectra from humic acid isolated from Ultisol (HAU), Inceptisol (HAI), sewage sludge (HAS) and vermicompost (HAV). The main absorption peaks are marked in the spectra

as well as the emergence of lateral roots (Fig. 2). The most effective HA (HAI; isolated from Inceptisol) induced about 50% more mitotic sites when compared to the most effective IAA concentration (10⁻¹⁰ M). In addition, root fresh weight was enhanced 244% by HA on average, while IAA (10⁻¹⁰ and 10⁻¹⁵ M) enhanced this feature only about 80% (Fig. 3a). Nevertheless, maize seedlings treated for 5 days with either HA or low IAA concentration exhibit increased proliferation of secondary roots (Fig. 2), resulting in enhanced root surface area (Fig. 3b). Although, under our experimental conditions, the number of MS and LRE is higher at the lowest IAA concentrations, the lateral root density

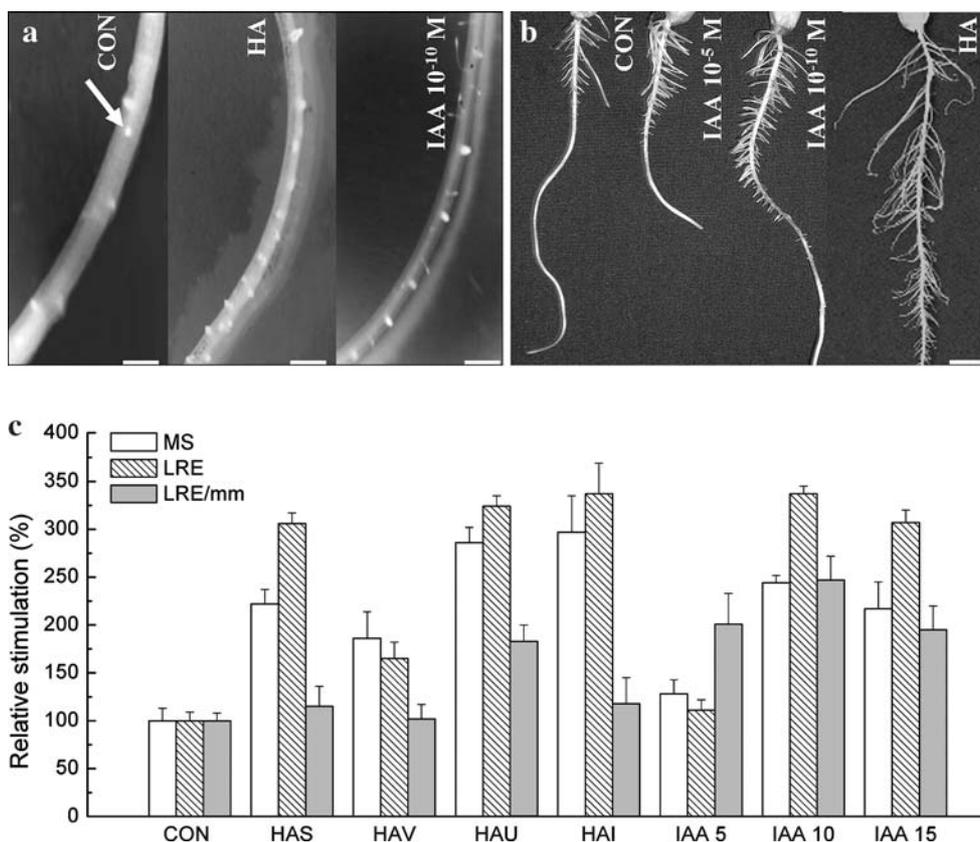


Fig. 2 a–c Effects of HA and IAA on maize root development evaluated by the induction of mitotic sites (MS) of lateral root emergence (LRE) and lateral root density (LRE per millimetre primary root length—LRE/mm). **a** Representative images of mitotic sites (arrow) of a control root, bar 0.8 mm; hyperinduction of mitotic sites in a root treated with HAU (20 mg C l⁻¹), or in a root treated with IAA (10⁻¹⁰ M), bars 1.2 mm. **b** Representative

roots treated with IAA (0, 10⁻⁵, 10⁻¹⁰ M) or HAU (20 mg C l⁻¹), bar 10 mm. **c** Quantification of early mitotic sites (clear columns), lateral root emergence (dashed columns) and lateral root density (grey columns). The data represent means from four independent experiments with ten plants analysed per treatment (±SD, n = 40)

(LRE/mm) remains quite similar for all IAA concentrations. On the other hand, the lateral root density hardly changed in most HA treatments unless for HAU which promoted a similar effect to that of IAA. Interestingly, the lowest IAA concentrations (10⁻¹⁰ and 10⁻¹⁵ M) also stimulated the elongation of primary roots even though a typical inhibitory effect was found in concentrations above 10⁻⁶ M (Fig. 3c). The data suggest that exogenous auxins at concentrations as low as 10⁻¹⁵ M IAA can also induce maize root growth but in a different way from that previously reported for a higher range of concentration (Mulkey et al. 1982; Zhao et al. 2002). It is worth noting that concentrations as low as 10⁻¹⁵ M IAA have never been tested before as far as it concerns the root growth process.

Effects of HA and IAA on proton pumps

Maize roots treated for 5 days with either HA or IAA exhibited a differential activation of their plasma

membrane and vacuolar H⁺-pumps (the vanadate-sensitive P type H⁺-ATPase, the concanamycin-sensitive V-ATPase and the K⁺-activated H⁺-PPase). This stimulation was observed in both activities, the substrate hydrolysis (Table 2) and ATP or PPI-dependent H⁺-pumping (Fig. 4). In most cases HA and IAA treatments could stimulate more the rates of H⁺ pumping activity (Figs. 4, 5) than the related ATP and PPI hydrolysis (Table 2). For instance, HAI could stimulate about sixfold more the initial rate of the H⁺ pumping through the plasmalemma than its related ATP hydrolysis, while HAU could enhance four to five times more the initial rates of vacuolar H⁺ pumping than the related ATP and PPI hydrolysis. Thus, it is tempting to speculate that at least part of the effect of HA as well as IAA treatments could be due to an increase in coupling of these H⁺ pumps. In addition, taking into account only the tonoplast H⁺ pumping activity (Fig. 5), some HA have affected preferentially the H⁺-PPase (HAV) or V-ATPase activity (HAI),

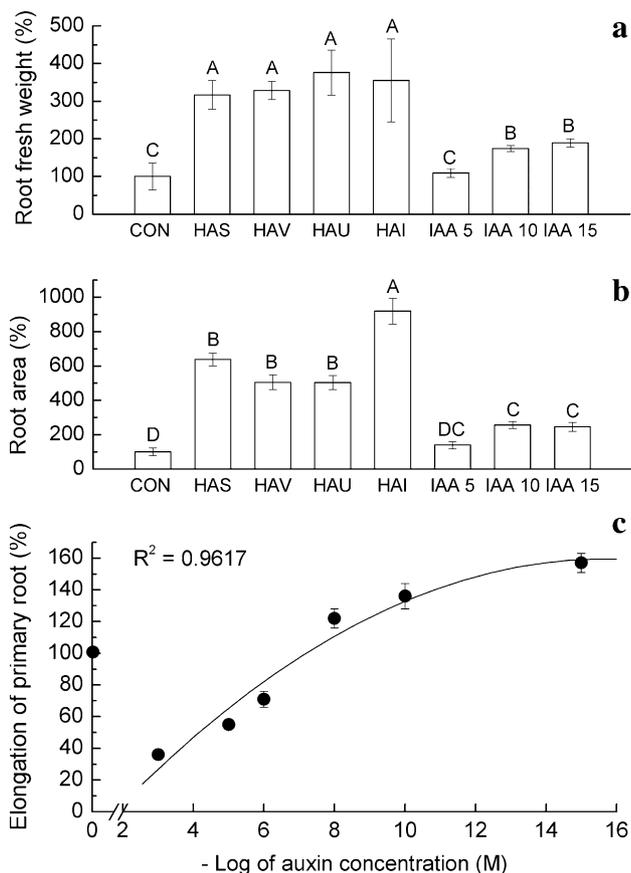


Fig. 3 a–c Effect of 20 mg C l⁻¹ HA from Ultisol (HAU), Inceptosol (HAI), sewage sludge (HAS) and vermicompost (HAV) or 10⁻⁵, 10⁻¹⁰ and 10⁻¹⁵ M IAA on roots fresh weight (a) and root area (b) of maize seedlings analysed by *DeltaTscan*[™] software. c Rate of primary-root elongation as a function of IAA concentration (from 10⁻³ to 10⁻¹⁵ M). Data represent means from four independent experiments performed with ten maize seedlings per treatment. Different letters in bars indicate statistical differences (Tukey test *P* < 0.05)

while others have activated both (HAU and HAS). A concentration-dependent behaviour was found in IAA treatments, indicating that such differential activation promoted by different HA could be due to either their specific concentration of hormone-like molecules or specific capability for releasing them. The effect of IAA on the H⁺-pumps activity was observed over a range of IAA concentrations and a dose–response relationship was plotted in a polynomial fit (Fig. 6). The plasmalemma and vacuolar ATPases had a similar behaviour, exhibiting a peak of stimulus at IAA 10⁻¹⁰ M and decreasing at lower concentrations (Fig. 6a, c). On the other hand, the H⁺-PPase stimulation started since 10⁻³ M and increased until 10⁻¹⁵ M (Fig. 6b). Although, there is no previous report on the effect of such a low concentration in plant physiology, Li and coworkers (2005) have demonstrated that

H⁺-PPase (AVP1) plays a key role in the auxin fluxes that regulate organogenesis. Thus, it is perhaps not surprising that this pump could be more sensitive to auxin than plasmalemma and vacuolar ATPases.

In order to investigate possible relationships between the HA chemical characteristics with the H⁺-pumps activities and other parameters related to their bioactivity such as root area, number of mitotic sites and lateral root emergence, a correlation analyses was carried out (Table 3). Total acidity (TA) and carboxylic acidity (CA) was directly correlated with vacuolar H⁺-ATPase, while it was inversely correlated with plasma membrane H⁺-ATPase and root area (RA) stimulation. The H/C was also directly correlated with vacuolar H⁺-ATPase activation and indirectly correlated with RA stimulation. The E₄/E₆ ratio and phenolic acidity (PA) were inversely correlated with H⁺-PPase, mitotic sites (MS) and lateral root emergence (LRE) stimulation (Table 3).

Discussion

An optimal root system is a key requirement for a plant's ability to survive adverse conditions and therefore, the lateral root number and placement is dramatically influenced by external factors (Leyser and Fitter 1998). The present work shows that exogenous IAA as well as HA can induce new sites of lateral root emergence, suggesting that even cells that were not endogenously preselected are capable of being recruited to the lateral root program by different sources of exogenous auxin-like growth regulators. Some evidences have shown that additions of auxins to the growth media can enhance lateral root formation (Blakely et al. 1988; Evans et al. 1994; Malamy and Ryan 2001), as well as a critical role for endogenous auxin has been described to the process of lateral root initiation (Casimiro et al. 2001; Bhalerao et al. 2002). Since the beginning of the last century soil humic substances have been studied as environmental agents endowed with auxin-like activities (Bottomley 1917). Thus, these data on HA-induced root branching and growth highlights these humic substances as potential factors by which root architecture is subject to regulation by environment.

Furthermore, this work also addressed the mechanism related to this lateral root proliferation, by providing evidences that exogenous IAA and different HA also stimulated the H⁺-pumps not only from plasmalemma but also from vacuolar membranes. Previously, most attention has been directed to only one mechanism for auxin-regulated plant growth involving directly H⁺-pumps modulation. This is based

Table 2 Effect of HA from Ultisol (HAU), Inceptisol (HAI) sewage sludge (HAS) and vermicompost (HAV) or 10^{-5} , 10^{-10} and 10^{-15} M IAA on proton pumps

Treatments	Hydrolytic activities		
	Plasma membrane	Tonoplast	
	H ⁺ -ATPase ($\mu\text{mol Pi mg}^{-1} \text{min}^{-1}$)	H ⁺ -ATPase ($\mu\text{mol Pi mg}^{-1} \text{min}^{-1}$)	H ⁺ -PPase ($\mu\text{mol PPI mg}^{-1} \text{min}^{-1}$)
Control	1.57 ± 0.13 (C)	0.15 ± 0.02 (C)	0.13 ± 0.02 (C)
HAU	2.75 ± 0.14 (B)	0.41 ± 0.02 (A)	0.32 ± 0.02 (A)
HAI	4.65 ± 0.24 (A)	0.24 ± 0.03 (BC)	0.29 ± 0.04 (AB)
HAS	4.58 ± 0.12 (A)	0.32 ± 0.02 (AB)	0.19 ± 0.01 (C)
HAV	2.97 ± 0.13 (B)	0.40 ± 0.03 (A)	0.16 ± 0.02 (C)
IAA 10^{-5} M	1.19 ± 0.17 (C)	0.23 ± 0.03 (BC)	0.17 ± 0.01 (C)
IAA 10^{-10} M	2.52 ± 0.12 (B)	0.33 ± 0.01 (AB)	0.18 ± 0.01 (C)
IAA 10^{-15} M	2.41 ± 0.10 (B)	0.17 ± 0.01 (C)	0.22 ± 0.01 (BC)

Vanadate- and concanamycin-sensitive ATP hydrolysis were measured in plasma membrane and tonoplast vesicles, respectively. The K⁺-stimulated pyrophosphate hydrolysis was analysed only in tonoplast ones. The vesicles were isolated from maize roots treated with 20 mg C l⁻¹ HA for 5 days. The reaction medium contained 50 mM Mops-Tris (pH 6.5 for plasmalemma or 7.0 for tonoplast), 3 mM MgSO₄, 100 mM KCl and 1 mM ATP or 0.1 mM PPI. Data represent means from four independent experiments ±SE. Different letters in columns indicate statistical differences (Tukey test $P < 0.05$)

on auxin-dependent activation and induction of de novo synthesis of the plasma membrane H⁺-ATPase, enhancing the apoplast acidification, which in turn, is essential for the activation of enzymatic cell wall plasticity (classical acid-growth theory, Hager et al. 1991; Rayle and Cleland 1992). A similar mechanism has been described to the bioactivity of humic substances, which can also stimulate the root growth underlying an induction and activation of the plasma membrane H⁺ pump (Canellas et al. 2002; Façanha et al. 2002; Quaggiotti et al. 2004). Here we provide some sound evidences for the involvement of vacuolar H⁺ pumps in the cellular strategies of root development, supporting the notion that the vacuole acts in combination with the cell wall dynamics to generate the cell turgor, the driving force for hydraulic stiffness and growth (Maeshima et al. 1996).

To the best of our knowledge, there is only one study (Pinton et al. 1992) that previously investigated the effects of humic substances on tonoplast H⁺-pumps. These authors observed that pre-incubation of tonoplast vesicles from oat roots with low molecular size humic substances (<5 kDa, essentially fulvic acids) could stimulate the activity of the vacuolar H⁺-ATPase. However, they concluded that this in vitro effect will be related to increased membrane permeability and disturbances on electrical membrane potential ($\Delta\psi$). It seems unlikely that this occurs when plants are exposed to HA, at least under our experimental condition (in vivo treatment), where the pump's activation could not be explained by only a disturbance on $\Delta\psi$, since all measurements were performed in the presence of 100 mM KCl. This is a physiological condition

in which the $\Delta\psi$ component is dissipated, enhancing the significance of the chemical (ΔpH) component to the H⁺ gradient formation (Bennett et al. 1984). Moreover, there was no evidence for increased leakiness as proton-tight vesicles were obtained from HA-treated roots, which could maintain high and stable H⁺ gradients (Fig. 4).

There are also only a few studies evaluating the effect of IAA on tonoplast H⁺-pumps. In vitro analyses have demonstrated that the addition of low IAA concentrations (10^{-6} – 10^{-10} M) to the tonoplast vesicles from red beet roots could also stimulate the hydrolytic activity of vacuolar pumps (Ozolina et al. 1996; Salyaev et al. 1999). Our data show a close correlation with these unique in vitro studies, since ATP and PPI hydrolysis changes in a similar IAA concentration-dependent way (Table 2). It is likely that environment-derived auxins may enter into maize root cells just as the endogenous one, and could access directly the pumps or indirectly through putative tonoplast intrinsic receptors. Furthermore, the H⁺-PPase is apparently much more sensitive to the IAA than the V-ATPase (Figs. 5, 6). It is possible that each pump could be activated by auxins in different magnitude as they do, responding to different physiological and stress conditions (Façanha and de Meis 1998). In addition, the H⁺-PPase activation and enhancement of emerged lateral roots promoted by HA were both inversely correlated with E₄/E₆ ratio (Table 3). This suggests that as higher is the humification degree of a HA as higher will be their bioactivity, and highlights the relevance of the tonoplast H⁺-PPase to lateral root initiation process. This is in line with recent data from

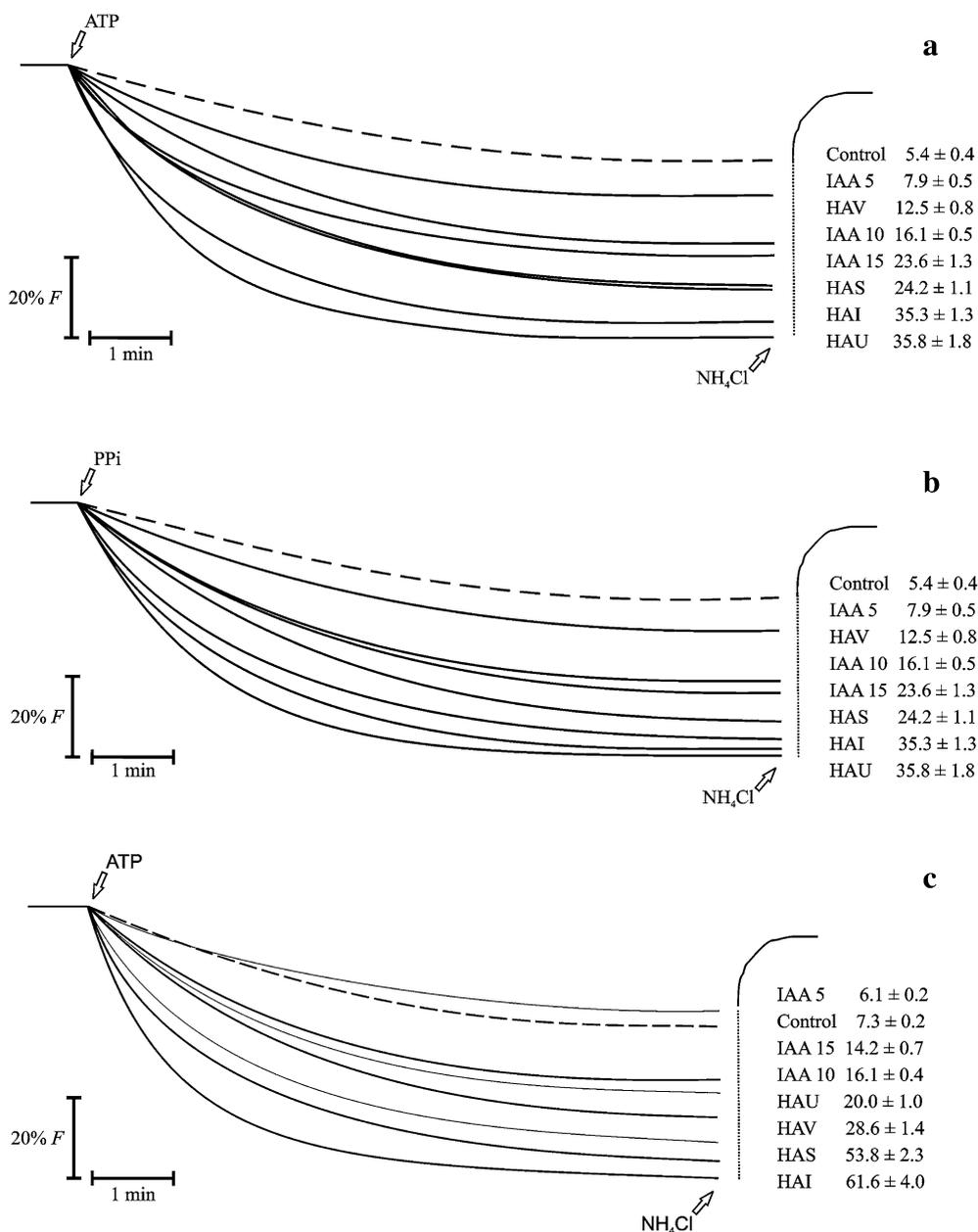


Fig. 4 a–c Proton gradient formation by vacuolar H⁺-ATPase (a), H⁺-PPase (b) and plasma membrane H⁺-ATPase (c) from maize roots treated with 20 mg C l⁻¹ HA from Ultisol (HAU), Inceptisol (HAI) sewage sludge (HAS) and vermicompost (HAV) or 10⁻⁵, 10⁻¹⁰ and 10⁻¹⁵ M IAA (IAA 5, IAA 10 and IAA 15, respectively). The reaction medium contained 10 mM Mops–Tris (pH 7.0 for tonoplast vesicles and pH 6.5 for plasma membrane),

3 mM MgSO₄, 100 mM KCl, 2.0 μM ACMA and 0.05 mg ml⁻¹ vesicle protein. The reaction was started by addition of 1 mM ATP (or 0.1 mM Ppi) and was carried out at 30°C. Once reached the steady-state, NH₄Cl was added to dissipate the H⁺ gradient. The figure shows normalised curves and their respective initial rates in ordered alignment in the side tables, which represent means from at least three independent experiments ±SE)

Li and co-workers (2005), which have elegantly demonstrated a role for H⁺-PPase AVP1 in auxin transport and auxin-related developmental processes such as root growth and morphology. However, these authors have focused mainly on the influence of AVP1 overexpression on the endogenous IAA transport, while a role for the V-ATPase in the acid growth process was only barely addressed. Clearly, the Figs. 5, 6

show that same concentration of IAA affect differently each of tonoplast H⁺-pumps. Although not examined in detail here, we predict that a differential V-ATPase activation and/or accumulation will also precede the IAA-induced growth responses, such as described for the H⁺-PPase, but during different environmental/physiological conditions signaled by distinct auxin concentrations.

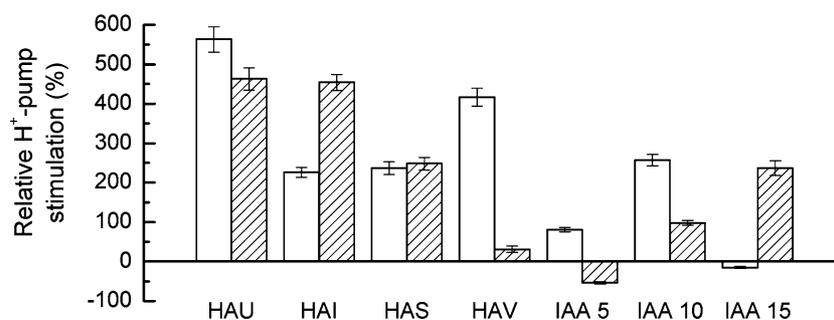


Fig. 5 Comparative analyses of HA and IAA effects on H⁺ pumping activities of V-ATPase (clear columns) and V-PPase (dashed columns). The treatments were respectively: 20 mg C l⁻¹ HA from Ultisol (HAU), Inceptsol (HAI) sewage sludge (HAS) and

vermicompost (HAV); and IAA 10⁻⁵, 10⁻¹⁰ and 10⁻¹⁵ M. Data represent percentages of stimulus calculated from the initial rates of H⁺-gradient data from Fig. 4

Indeed, there are compelling evidences for participation of the tonoplast H⁺-ATPase in the plant growth process. It was suggested that both tonoplast pumps could function on formation of osmotic potential needed for expanding cells (Maeshima et al. 1996; Smart et al. 1998). Gogarten and co-workers (1992) have shown that antisense inhibition of V-ATPase subunit A reduced cell expansion in transgenic carrot lines. It was also reported analysis of an *Arabidopsis* (det3) mutant with reduced V-ATPase activity and retarded elongating hypocotyls together with studies using concanamycin A, a specific inhibitor of V-ATPases (Schumacher et al. 1999). More recently, the description of a function of VHA-c1 gene expression in expanding organs of etiolated seedlings, and elongation zone of roots have confirmed that this highly conserved eukaryotic H⁺ pump plays an important role in plant cell expansion (Padmanaban et al. 2004).

The data also reveal that HA seems to be much more effective than any of the IAA concentrations tested. Root fresh weight was also enhanced by more than three times in average by HA, but IAA treatments have only slightly modified this feature (Fig. 3a). Although the lateral root's emergence was stimulated nearly equally by both HA and IAA treatments

(Fig. 2), HA enhanced root area up to 540% on average (Fig. 3b), whereas the most effective IAA concentration (10⁻¹⁰ M, Fig. 3b) stimulated it by only 150%. It seems likely that root development is highly stimulated by HA (Fig. 3) mainly due to a faster elongation of their primary and lateral roots rather than a higher lateral root density (LRE/mm) as occurs with IAA treatments (Fig. 2b). Although the number of MS and LRE are stimulated by all HA treatments and at the lowest IAA concentrations, the lateral root density was stimulated almost equally in all IAA concentrations while this parameter hardly changed in most HA treatments unless for HAU which promoted an effect similar to that of IAA (Fig. 2c). These data imply that different HA can influence differently the root architecture and are also consistent with the notion that IAA concentrations detected in humic substances hardly justified their biochemical activities (e.g., Muscolo et al. 1998; Pizzeghello et al. 2001). Perhaps humic substances can promote root development by involving more than one phytohormone. For instance, auxin regulation of root development is also dependent on endogenous ethylene balance (Stepanova et al. 2005). On the other hand, there are more than 50 different compounds, which can express auxin activity (Bertosa et al. 2003;

Table 3 Pearson correlation coefficients between some chemical characteristics of the HA [elemental ratios (H/C, O/C, C/N), total acidity (TA), carboxylic acidity (CA), phenolic acidity (PA), E₄/E₆ ratio (E₄/E₆)], and some parameters related to their bioactivity

	P-ATPase	V-ATPase	H ⁺ -PPase	RA	MS	LRE
H/C	-0.4319	0.7095*	-0.2686	-0.8383*	-0.4308	-0.1636
O/C	-0.7782*	0.8467*	0.5884	-0.5848	0.3505	0.1650
C/N	-0.5232	0.2270	-0.4663	-0.3025	-0.5504	-0.8879*
TA	-0.7864*	0.9187*	0.4122	-0.7356*	0.1644	0.0789
CA	-0.7604*	0.8975*	0.4538	-0.7017*	0.2115	0.1287
PA	-0.2444	0.0401	-0.8264*	-0.2903	-0.8426*	-0.9594*
E ₄ /E ₆	-0.2267	0.0534	-0.8744*	-0.3295	-0.8901*	-0.9609*

*Significant coefficient at $P < 0.001$

[root area (RA), number of mitotic sites (MS), lateral root emergence (LRE) and proton pumps activities (P-ATPase, V-ATPase, H⁺-PPase)]

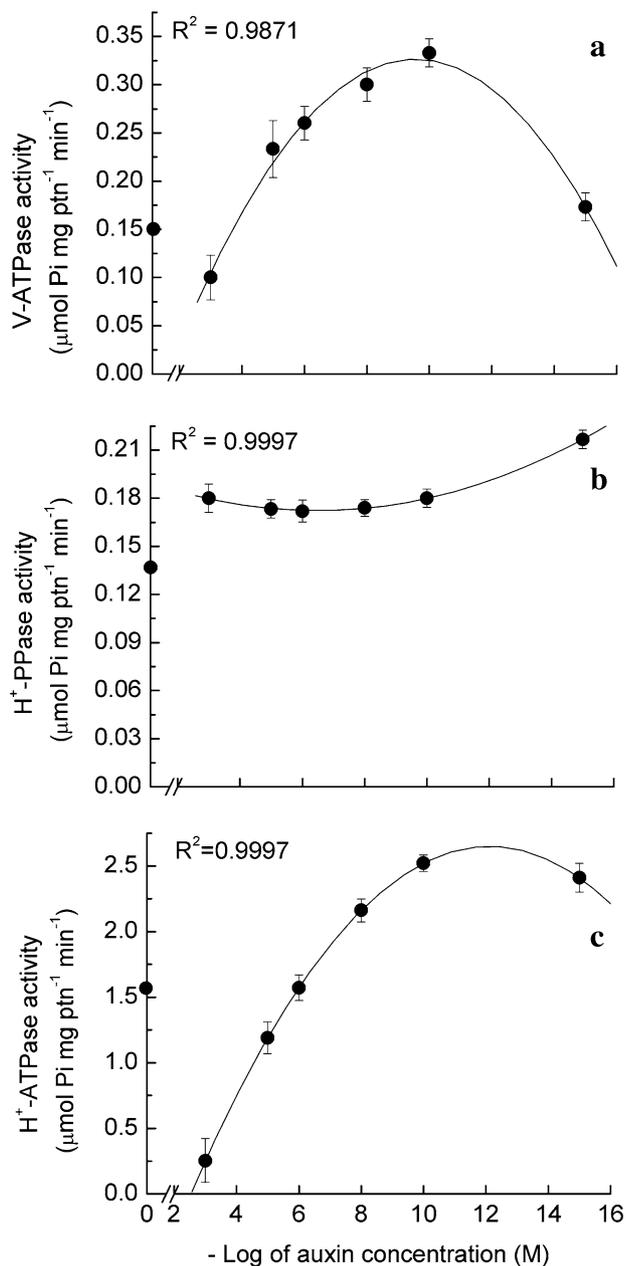


Fig. 6 a–c Hydrolytic activity of the proton pumps as a function of IAA concentration. Concanamycin-sensitive V-ATPase activity (a) and K⁺-stimulated H⁺-PPase activity (b) of tonoplast vesicles and vanadate-sensitive P-ATPase activity (c) of plasma membrane vesicles. Polynomial fit were calculated from the data which represent means ±SD of three independent experiments

and references therein). In the light of this, the significance of HA–IAA groups in explaining the whole HA bioactivity should be regarded with caution, since this could be accounted for other bioactive molecules still unidentified in the HA endowed with such hormonal activity. Furthermore, effectiveness of HA in regulation of root development also depends on dynamics of releasing of such bioactive molecules from their macro-

structure, which in turn is related to synergic and antagonic interactions occurring in rhizosphere between the root exudates and humic substances.

Uncertainty regarding the mechanism by which humic substances stimulate plant biochemical activities is also in part due to their heterogeneity and the difficulty of their characterization (Pizzeghello et al. 2001). Therefore, we performed a structural analysis of HA by FT-IR spectroscopy aiming to shed light on the relationships among these structural aspects, bioactivities and sources from which each HA was isolated. FT-IR spectroscopy is a very useful tool to analyse organic compounds especially in the region of absorption between 1,492 and 900 cm⁻¹ described as a finger print region (Coulthup et al. 1964). Each HA presented peculiar absorptions in this region and they exhibited a wide range of elemental composition, total acidity and E₄/E₆ ratio (Table 1). In spite of such structural differences, all of these HA have stimulated the lateral root development as well as the activities of the plasma-lemma and tonoplast H⁺ pumps, although the magnitude of stimulus has varied in each case.

Humic substances' action on plant growth is complex, and previous studies on relationships between their chemical composition and bioactivity have produced contradictory results (Mato et al. 1972; Vaughan et al. 1985). The magnitude of the effects on tonoplast H⁺ pumps activities was dependent of the source from which each HA was isolated and, consequently, also from their chemical nature. Soils with higher weathering degree (e.g., Ultisols) presented HA with higher values of O/C, and total and COOH acidities as a result of less humified organic matter. This is in agreement with the general humification theory (Stevenson 1994), which postulates the presence of humic material more oxidised in soils with highest weathering degree. Canellas and Façanha (2004) found that more condensed humic substances can promote highest stimulation of the microsomal H⁺-ATPases from maize roots. The generation of vermicompost and sewage sludge is characterised by strong biological activity occurring in the first step of humification process, resulting in the stabilisation of organic residues and formation of the HA, which exhibit a strong influence on the root development (Table 2, Figs. 2, 3). Here, more condensed HA also promoted a higher stimulation of both tonoplast H⁺ pumps and maize root growth, since higher bioactive HA were isolated from soils of lowest weathering degree (HAI).

Taking together the results presented here and previously published data, we postulated a still unexplored mechanism of action for the hormonal activity of environmentally derived indolacetic acid and HA,

involving a concerted activation of the plasmalemma and tonoplast H^+ pumps. Such phenomenon can be found in HA derived from anthropogenic as well as natural sources of humic substances. These findings depict a tempting scenario for further biochemical and molecular studies on hormonal regulation of vacuolar and cell membrane electrochemical gradients and their role in the cell expansion mechanism involved in the root growth and development.

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