Humic Acids Isolated from Earthworm Compost Enhance Root Elongation, Lateral Root Emergence, and Plasma Membrane H^+-ATPase Activity in Maize Roots[1]

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Earthworms (*Eisenia foetida*) produce humic substances that can influence plant growth by mechanisms that are not yet clear. In this work, we investigated the effects of humic acids (HAs) isolated from cattle manure earthworm compost on the earliest stages of lateral root development and on the plasma membrane H^+-ATPase activity. These HAs enhance the root growth of maize (*Zea mays*) seedlings in conjunction with a marked proliferation of sites of lateral root emergence. They also stimulate the plasma membrane H^+-ATPase activity, apparently associated with an ability to promote expression of this enzyme. In addition, structural analysis reveals the presence of exchangeable auxin groups in the macrostructure of the earthworm compost HA. These results may shed light on the hormonal activity that has been postulated for these humic substances.

Earthworm (*Eisenia foetida*) compost strongly affects soil fertility by increasing availability of nutrients, improving soil structure and water-holding capacity (Landgraf et al., 1999). It has been suggested that earthworms can increase the velocity of decomposition of organic residues (Vinceslas-Akpa and Loquet, 1997) and also produce several bioactive humic substances (e.g. Cacco and Dell'Agnola, 1984; Nardi et al., 1994; Masciandaro et al., 1999). These substances are endowed with hormone-like activity that improves plant nutrition and growth (Vaughan and Malcolm, 1985; Chen and Aviad, 1990). Humic acids (HAs) comprise one of the major fractions of humic substances. They are characterized by dark-colored, alkali-soluble, acid-insoluble, and high-M_r humified organic matter (Schnitzer, 1991). In fact, it has been known since the early 1900s that HA can display auxin-like activities (Bottomley, 1917; Dell'Agnola and Nardi, 1987; Muscolo et al., 1999). In turn, it has been proposed that one of the mechanisms by which auxins can stimulate plant growth is by inducing an increase in the amount of plasma membrane (PM) H^+-ATPase, which acidifies the apoplast and thereby loosens the cell wall, allowing cell elongation (Hager et al., 1991; Frias et al., 1996). Activation of the H^+-ATPase can also improve plant nutrition by enhancing the electrochemical proton gradient that drives ion transport across the cell membrane via secondary transport systems (for review, see Sze, 1985; Morsomme and Boutry, 2000).

Previous reports have demonstrated that some low-M_r humic substances (essentially fulvic acids) can stimulate the H^+-ATPase of PM vesicles isolated from roots of several plants. This effect was attributed to a dissipation of the electrical potential and an increase in membrane permeability (Nardi et al., 1991; Varanini et al., 1993) or to enzyme modulation by an undefined posttranslational mechanism (Pinton et al., 1999; Nardi et al., 2000). Although a large body of evidence indicates that HA can also directly affect enzymatic activities in several metabolic pathways (Vaughan and Malcolm, 1985), relatively little attention has been paid to the biochemical effects of the HA fraction on plant metabolism and development.

In the present work, the PM H^+-ATPase activity and the induction of mitotic sites associated with the earliest stages of lateral root development have been investigated as a basis for analysis of a possible hormonal effect of earthworm compost HA on lateral root proliferation.

RESULTS

HA Structural Features

According to elemental composition analysis of HA, the values for total carbon, oxygen, nitrogen,

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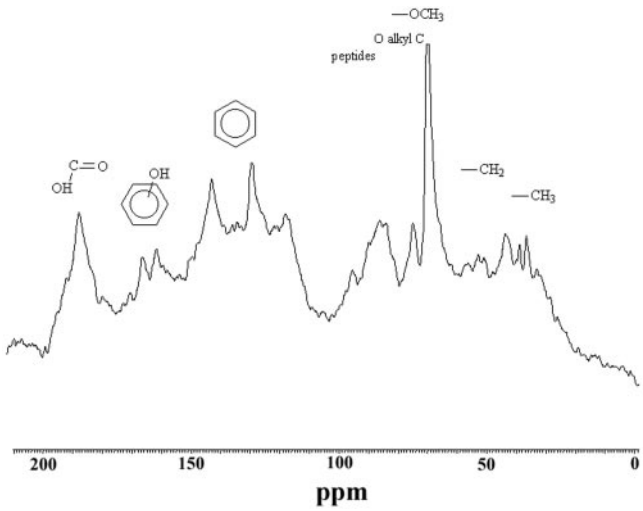


Figure 1. ^{13}C NMR spectrum from HA extracted from earthworm compost. The spectrum is divided into regions corresponding to specific chemical classes: aliphatic C, 0 to 50; *N*-alkyl and methoxyl C, 50 to 60; aliphatic C-O (carbohydrates), aromatic C, 110 to 145; phenolic C, 150 to 165; and carboxyl, esters, and amides C, 165 to 190.

hydrogen, and ash were 48.5%, 42.2%, 3.2%, 5.6%, and 0.51% (on a dry weight basis), respectively. The carboxyl and phenolic groups account for 496 and 170 centimol charge kg^{-1} of the total acidity (666 $Cmol_c$ kg^{-1}). ^{13}C NMR analysis (Fig. 1) revealed weak absorption in the hybridization state of the atom C (sp^3) C atom region (δ_C-0–40) because of CH_2 and CH_3 groups in long alkyl chains. Around δ_C-15, the spectra showed signals characteristic of terminal methyl groups and between δ_C 20 and 30 due to the presence of $(CH_2)_n$ groups. Curiously, a very large peak was observed in the region δ_C-44 to -57, which is not typical of ^{13}C NMR spectra from pedogenic HA and, thus, revealed the low humification stage of HAs isolated from earthworm because of an increase in C bonded to mono- and di-O. The signals at δ_C-44, -53, and -55 can be attributed to sp^3 carbon atoms, including C bound to N in amino acids; at δ_C-57, they are because of OCH_3 groups bound to the hybridization state of the atom C (orbital sp^2 bound). The signal around δ_C-65 can be attributed to carbinolic C of primary alcohols and polysaccharides. The signal at δ_C-70.6 indicates sp^3 C atoms bound to N. The signals present around δ_C-100 suggest sp^3 carbon atoms bound to two atoms of oxygen (anomeric carbon), as found in carbohydrates. The peak centered at δ_C-130 is because of aromatic carbons. The high field peaks at δ_C-150 to -160 are because of carbon bonded to phenolic OH groups. The δ_C-160 to -190 region shows signals because of the presence of differently substituted carbonyl-C atoms. Quantitatively, the spectra revealed 8.7% carboxyl, 7.4% phenolic, 41.9% aromatic, 31.5% peptide and carbohydrate, and 17.5% other aliphatic carbons. In comparison with typical values reported for an average soil HA (Schnitzer, 1991), the earthworm compost HA had a low content of C in alkyl chains and carboxylic functions and a high content of aromatic and mono- or di-O-C alkyl and N-C atoms. However, this HA-earthworm compost fraction is endowed with the characteristic structural network described for most HAs isolated from different sources of organic matter (e.g. Clapp and Hayes, 1999). This is consistent with the notion that the same pathways for the formation of HA may operate in all environments resulting in a substance with defined identity (Stevenson, 1994).

Detection of 3-Indole Acetic Acid (IAA) in Earthworm Compost HA

The presence of IAA in the HA structure was identified by gas chromatography (GC)-mass spectrometry (MS; Fig. 2). To reduce the polarity, standard methylated IAA and methylated HA were produced, and revealed similar retention times of 7.48 to 7.51 min and mass spectra with the molecular ion $[M^+]^{\bullet}$ at 189 m/z with the base peak at 130 m/z. These data are compatible with the presence of the ion formed by fragmentation by elimination of the $-CO_2Me$ radical, confirming the presence of IAA in the HA structure. This is in agreement with recent reports on IAA detection by immunoassay in humic substances extracted from different sources (Muscolo et al., 1998; Pizzeghello et al., 2001). However, it is worth noting that in our case, a low-M_r molecule is detected here after dialysis of the HA fraction with a membrane having a molecular mass cutoff of 14 kD. These exchangeable IAA molecules have to be linked to the

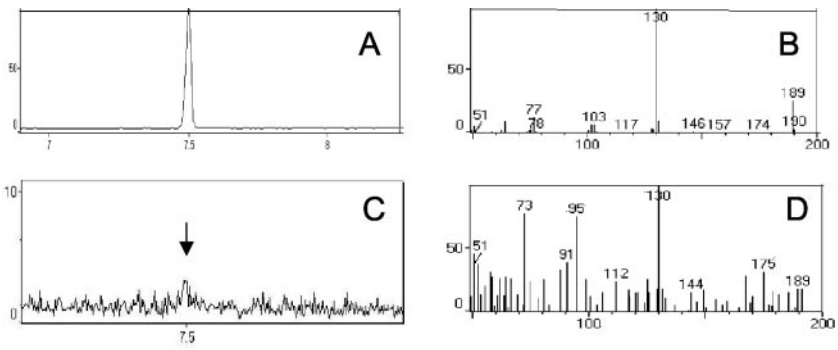


Figure 2. Gas chromatogram of the standard methylated IAA (A) with the respective mass spectra (B), and the gas chromatogram of the methylated HA isolated from earthworm compost (C) with the respective mass spectra (D).

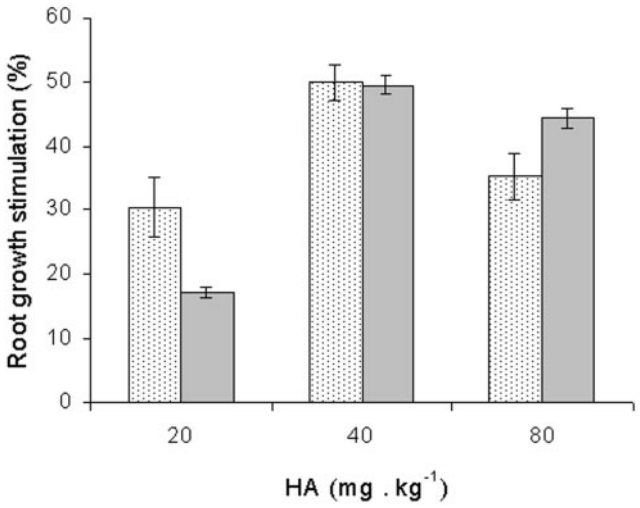


Figure 3. Effect of different HA concentrations on roots length (light columns) and radicular superficial area (gray columns) of maize seedlings analyzed by SIARCS software. Total root length was calculated as a sum of the length of primary and lateral roots. Data are expressed as percentage (±SE) of control (plants grown without HA) and represent normalized means from six independent experiments performed with maize seedlings (25 plants per treatment in each case).

HA macrostructure by means of hydrophobic interactions in clusters intrinsic to the supramolecular structure of HA (e.g. Schulten and Schnitzer, 1995; Piccolo, 2002).

Root Length and Surface Area

Total radicular length for each seedling was calculated as the sum of the lengths of all radicular nodal segments, using automatic linearization with SIARCS software (Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisa e Desenvolvimento da Instrumentação Agropecuária, São Carlos-SP, Brazil; Cruvinel et al., 1996). In maize (*Zea mays*) seedlings treated for 7 d with different HA concentrations, both elongation and proliferation of secondary roots were stimulated, resulting in enhanced root surface area (light columns in Fig. 3), and the increase in total radicular length (gray columns in Fig. 3).

HA-Induced Sites of Lateral Root Emergence

The elongation differentiation zone of the root includes small, densely meristematic cells that are in continuous metabolic activity and are more susceptible to lateral root formation. We have measured the proliferation of the mitotic sites in this zone of roots treated or not with 40 mg L^{-1} earthworm HA (Fig. 4). Despite the tendency for HA to induce more sites of lateral root emergence, no statistical significance was obtained until 3 d after maize root exposure to HA in the growth medium, when HA treatment clearly stimulated the number of sites of lateral root emergence to a level ranging from 7 to 12 times the control values. This marked effect on the root morphology was mainly observed at the elongation/differentiation zone (data not shown). The hyperinduction of sites of lateral root emergence after HA treatment can be observed in Figure 4B. Figure 4C shows a single mitotic site before lateral root emergence highlighted in an untreated root.

HA Effect on H^+-ATPase Activity

PM vesicles isolated from maize roots treated for 7 d with 40 mg L^{-1} HA exhibited a clear stimulation of the vanadate-sensitive ATPase activity (Fig. 5A) as well as of the formation of an ATP-dependent proton gradient, measured as a quenching of ACMA fluorescence (Fig. 5B). The initial rate of gradient formation and ATP hydrolysis were enhanced by 2- to 3-fold in response to treatment with earthworm compost HA. Interestingly, addition of HA to the reaction medium in vitro inhibited both the ATPase activity

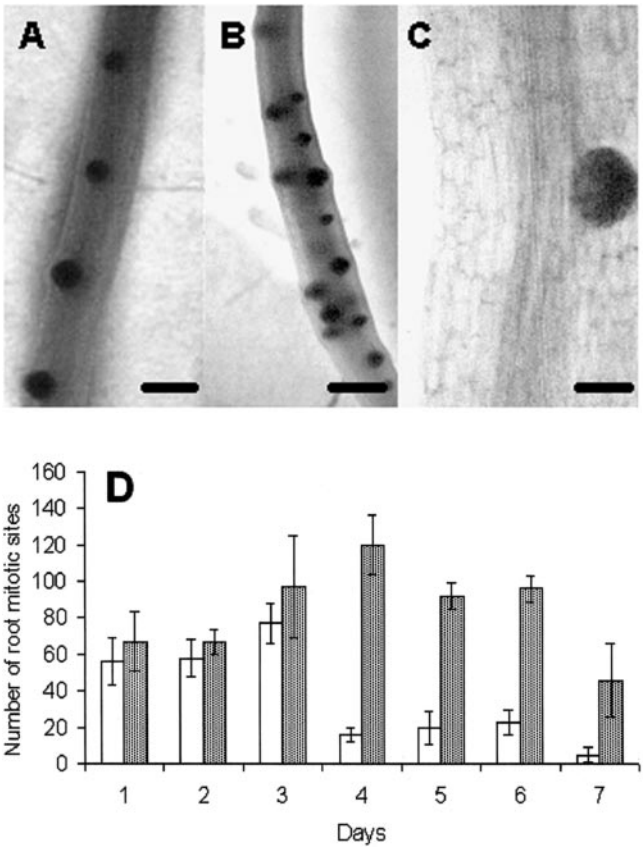
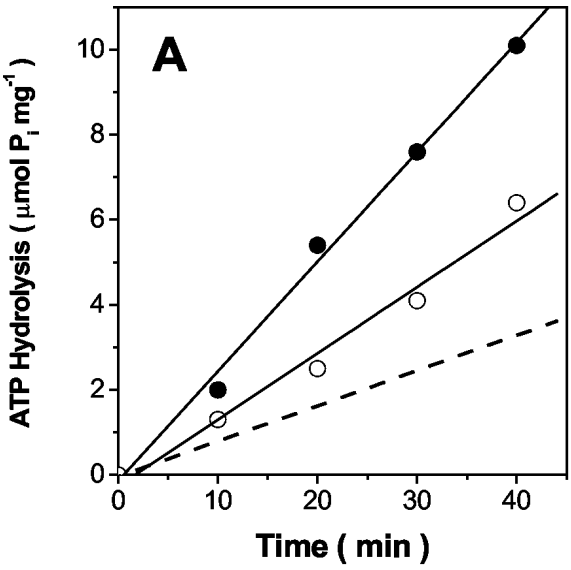


Figure 4. Effect of HA on the root growth pattern evaluated by quantification of lateral root mitotic sites. A, Control roots showing some mitotic sites. Bar = 1 mm. B, Hyperinduction of mitotic sites in a maize root after HA incubation (40 mg L^{-1}). Bar = 1 mm. C, Single mitotic site before lateral root emergence. Bar = 200 μm. D, Counting of root mitotic sites. Light columns represent control roots and gray columns represent HA-treated roots.



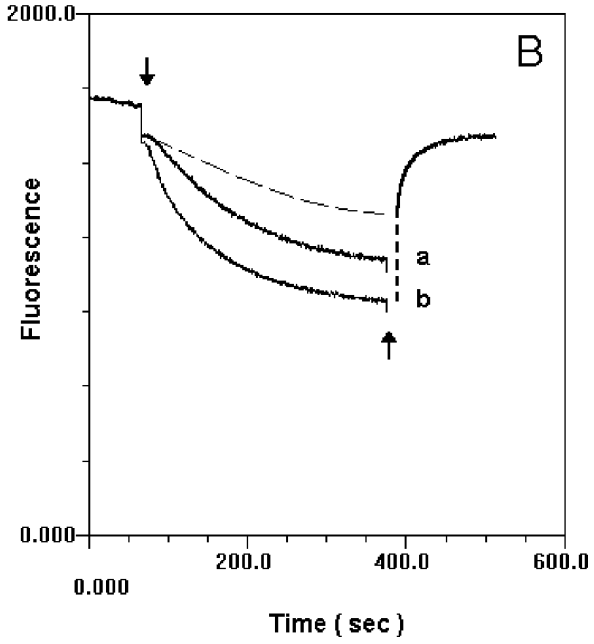


Figure 5. Effects of HA on PM H^+-ATPase activity and proton pumping. Vanadate-sensitive ATP hydrolysis (A) and proton transport (B) were measured in PM vesicles isolated from maize roots treated (λ) or not (μ) with 40 mg L^{-1} HA for 7 d. Dashed line shows the ATPase activity from control vesicles recorded in the presence of 40 mg L^{-1} HA in vitro. In A, the reaction medium contained 50 mM HEPES-KOH (pH 6.5), 3 mM $MgSO_4$, 100 mM KCl, and 1 mM ATP. In B, the same medium was used, except for the buffer concentration (10 mM HEPES-KOH) and the presence of 2.5 μM 9-amino-6-chloro-2-methoxyacridine (ACMA), a fluorescent pH probe. Arrows indicate the addition of 1 mM ATP to start the reaction and of 3 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, a protonophore, which dissipates the proton gradient. A and B are representative experiments of at least four independent preparations of PM from maize roots. Differences are significant at $P < 0.001$ (Student's *t* test).

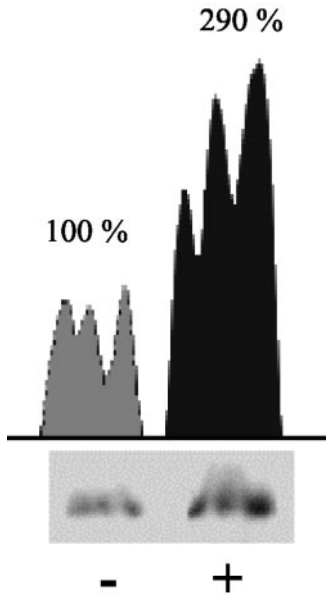


Figure 6. Western blot of PMs isolated from maize roots treated (+) or not (−) with 40 mg L^{-1} HA for 7 d. Fifty micrograms of vesicle protein was separated by 7.5% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots were probed with antibodies against PM H^+-ATPase and developed with peroxidase-conjugated secondary antibody. The immunoresponse was quantified densitometrically (upper) as described in "Materials and Methods."

as well as the proton gradient formation in PM-enriched vesicles isolated from roots grown under control conditions (dashed lines in Fig. 5). Western-blot analysis using antibodies raised against H^+-ATPase PMA2 isoform from *Nicotiana plumbaginifolia* Viv. (Morsomme et al., 1996) revealed that the amount of immunoreactive protein at the PMA locus (approximately 96 kD) also increased almost 3-fold in the membrane vesicles isolated from maize roots treated with HA (Fig. 6). It is worth noting that no change in total protein content was found because the yield from 20 g fresh weight was approximately 3 mg of protein for both control and treated preparations (data not shown). Taken together, these results suggest that earthworm compost HA treatment can affect the H^+-ATPase activity indirectly by promoting an increase in the concentration of H^+-ATPase in membrane vesicles.

DISCUSSION

Although the stimulatory effect of earthworm composts on plant development is attributed historically to their nutrient content, specific effects of the HA fraction from these composts on plant metabolism is usually unrelated to its ash content (Vaughan and Malcolm, 1985, and refs. therein). In this study, we explored the structure and function of the HA fraction isolated from an earthworm compost. This fraction induces the proliferation of sites of lateral root emergence in maize roots (Fig. 3). These differentia-

tion sites are precursors of lateral roots and are formed by meristematic cells that have a PM enriched with H^+-ATPases (Jahn et al., 1998). Therefore, it is possible that the enhancement of the PM H^+-ATPase content (Fig. 6) might be associated with the induction of mitotic sites by HA (Fig. 4). Because the total protein content of the PM-enriched fraction (relative to the fresh weight) was not significantly modified by HA treatment, it is likely that HA can enhance the expression of the PM H^+-ATPase gene. Furthermore, addition of HA to the reaction medium promotes inhibition of both ATP hydrolysis and H^+ transport (dashed lines in Fig. 5), suggesting that if HA could gain access to the cytoplasm, this enzyme probably would be inhibited. This may mean that the stimulatory effect of HA can be triggered by an association of these high-M_r molecules with specific receptors on the cell surface. Another possibility would be a release of small bioactive molecules from the HA macrostructure; these small molecules might interact with receptors on the PM or even inside the cell.

Using GC-MS, we have detected the presence of auxin groups in HA extracted from earthworm compost. Although HA are considered macromolecules consisting mainly of long alkyl chains containing aromatic groups, the occurrence of hydrophobic clusters in their supramolecular structural conformation also has been described (Schulten and Schnitzer, 1995; Clapp and Hayes, 1999). The presence of intrinsic small bioactive molecules such as IAA clustered within the HA supramolecular arrangement might be related to both the induction of root mitotic sites and H^+-ATPase activation. Previously, IAA was also detected by immunoassay in humic substances extracted from other sources (Muscolo et al., 1998). However, the separation of IAA from HA by GC means that this small molecule can be released by polarity changes in the HA microenvironment. In field conditions, such changes of polarity can occur by interactions between soluble HA and root exudates (Nardi et al., 2000; Cozzolino et al., 2001).

The phytohormone auxin is a key regulator of lateral root development (Blakely et al., 1982; Laskowski et al., 1995). It has been demonstrated that root basipetal and leaf acropetal auxin transport activities are required during the initiation and emergence phases, respectively, of lateral root development (Casimiro et al., 2001; Bhalerao et al., 2002). On the other hand, the acid growth theory postulates that the acidification of the apoplast caused by activation of the PM H^+-ATPase can induce cellular expansion (for review, see Rayle and Cleland, 1992). This phenomenon has been associated with an auxin-induced activation of the P-type H^+-ATPase by an as-yet-unclear mechanism (Morsomme and Boutry, 2000). It has been reported that auxin can induce de novo synthesis of the PM H^+-ATPase in plant tissues (Hager et al., 1991), correlated with an induction of H^+-ATPase mRNA of the major isoform (MHA2) expressed in maize (Frias et al., 1996). Although this effect was studied in coleoptiles, it has been shown that growth depends on extracellular pH in maize roots also (Peters and Felle, 1999, and refs. therein). Moreover, in maize protoplasts, a receptor that binds auxin from outside the PM was identified through which the H^+-ATPase could be activated (Ruck et al., 1993).

Taking our data together with those from the literature, it is tempting to speculate that, like endogenous auxins (Ruck et al., 1993; Goldsmith, 1993; Abel et al., 1994), HA-IAA groups may access plant receptors and trigger a cascade that activates transcription factors and protein synthesis and at the same time alters the activity of particular enzymes like the PM H^+-ATPase. The ultimate physiological response would involve a higher level of cell activity and tissue differentiation resulting in root growth. However, despite the observation that auxins and earthworm compost HA share such functional similarities, we cannot rule out the possibility that other bioactive groups that may be present in the complex HA structure can also contribute to induction of lateral root development and H^+-ATPase activation.

MATERIALS AND METHODS

Materials

A mixture of plant residues from *Panicum maximun* Jacq. and cattle manure 5:1 (v/v) was used for earthworm (*Eisenia foetida*) compost preparation. The organic residues were mixed and earthworms were added at a ratio of 5 kg worms per m^3 of organic residue. A bed of worms and organic residues was first prepared in a container and additional layers of organic residue were subsequently distributed over the pile at times depending on the temperature until the pile reached 50 cm. At the end of transformation process (3 months after distribution of the last organic residues), the worms were removed by placing a pile of fresh organic residue (plant + cattle manure) in a corner of the container. The organic matter composition of the earthworm compost was: pH 6.2, 134 g kg^{-1} total organic carbon, 13.3 g kg^{-1} total nitrogen, 10:1 C:N ratio, and 16.4 g HA carbon kg^{-1}.

Extraction of HAs

The humic substances were extracted as described by the International Humic Substance Society (Schnitzer and Skinner, 1982). In brief, 10 volumes of 0.5 mol L^{-1} NaOH was mixed with 1 volume of earthworm compost, under N_2 atmosphere. After 12 h, the suspension was centrifuged at 5,000*g* and acidified to pH 1.5 using 6 mol L^{-1} HCl. The solubilization and precipitation of HA were repeated three times and the last pellet was mixed with 10 volumes of a diluted mixture of HF-HCl solution (5 mL L^{-1} HCl [12 M] + 5 mL L^{-1} HF [48%, v/v]). After centrifugation (5,000*g*) for 15 min, the sample was repeatedly washed with water until a negative test against $AgNO_3$ was obtained, followed by dialyzing against deionized water using a 12- to 14-kD cutoff membrane (Thomas Scientific, Swedesboro, NJ). The dialyzate was lyophilized and characterized chemically. Then, the HA powder was solubilized with 50 to 100 mL of 0.05 mol L^{-1} NaOH and the pH was adjusted to 5.5 with 0.1 M HCl.

HA Structural Features

The elemental composition was determined using a CHN Perkin-Elmer autoanalyzer (Perkin-Elmer, Foster City, CA). Total acidity [$Ba(OH)_2$ method] and carboxylic acidity [$Ca(Oac)_2$ method] were determined according to

Schnitzer and Gupta (1965) followed by a potentiometric titration. The phenolic acidity was obtained by the difference. The ^{13}C NMR spectra were run in solution using an AC-200 spectrometer (50.30 MHz for ^{13}C, Bruker Instruments, Billerica, MA), and 150 mg of HA dissolved in 1 mL of NaOH (0.5 mol L^{-1}) containing 9:1 (v/v) water:D_2O. The analysis was performed using the INVGATE sequence (inverse decoupling), with a pulse of 90°, acquisition time of 0.2 s, 16 K of size, and about 200,000 transients for each sample. The chemical shift ($\delta^{13}C$) was expressed on a scale relative to tetramethylsilane ($\delta^{13}C = 0$).

Detection of IAA in HA

Methylated HA was prepared by treatment with acetyl chloride in methanol suspension HA (30 mg dry weight) and methanol (7 mL) were mixed in a 100-mL flask in an ice bath. One milliliter of acetyl chloride was slowly added to the resulting suspension. The flask was shaken for 4 h at 25°C. This procedure was repeated three times, except that the last reaction was carried out overnight. The suspension was centrifuged for 30 min at 2,680*g*. The clear brown solution was dried in a rotary evaporator and dissolved in 10 mL of methanol. The methylated HA solution was analyzed by GC-MS (QP5050A equipped with DB1 column, 30-m × 0.25-mm i.d., Shimadzu, Columbia, MD). The oven temperature was programmed to increase from 100°C to 280°C at a rate of 15°C min^{-1}. Helium at a flow rate of 1 mL min^{-1} was used as carrier gas. The ionizing voltage of the MS was 70 eV. Pure IAA from Sigma (St. Louis) was methylated as described above for HA and subjected to GC-MS. IAA detection in the chromatogram of methylated HA was accomplished by comparing retention time and mass spectra with those of the purified methylated IAA sample.

Plant Growth and HA Treatment

Maize (*Zea mays* var. BR 106) seeds provided by Empresa Brasileira de Pesquisa Agropecuária (Seropedica, Brazil) were surface sterilized by soaking in 0.5% (w/v) 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 approximately 0.5 cm long were transferred into a solution containing 2 mM $CaCl_2$ and either 0, 20, 40, or 80 mg dry weight L^{-1} HA extracted from earthworm compost. Previous experiments showed that seedlings treated for 7 d with 40 mg dry weight L^{-1} exhibited the maximum rate of relative root elongation (data not shown). It is worth noting that HA can improve the plant growth in water as well as in complete nutrient solution. The stimulation (in water) in the presence of HA exceeded that obtained in nutrient solution (for review, see Chen and Aviad, 1990). A minimal medium ($CaCl_2$, 2 mM) has been used in this work to avoid any interference from nutrient constituents that could function synergistically along with HA on plant growth and metabolism (e.g. Pinton et al., 1999).

Root Growth Measurement

On the 7th d, the roots were collected to estimate their lengths and areas using SIARCS software image analyzer (Cruvinel et al., 1996). Other samples of root seedlings were collected and used for further experiments.

Frequency of Sites of Lateral Root Emergence

Seeds of maize were germinated for 4 d in wet filter paper and rooted in a medium containing 0 or 40 mg L^{-1} of HA. The whole root systems (three replicates) of both treatments were harvested every day during a period of 7 d to evaluate the number of mitotic sites, as follows: The entire root system was washed in water and cleared by boiling at 75°C for 20 min in KOH (0.5%, w/v). Afterward, root samples were rinsed in water and stained for 14 h in the dark in hematoxylin staining solution. Then, they were rinsed in water and destained in 80% (w/v) lactic acid at 75°C for 30 to 90 s. Individual specimens were transferred to petri plates containing water and 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. Hematoxylin stock solution consisted of 1 g hematoxylin, 0.5 g ferric ammonium sulfate, and 50 mL of 45% (w/v) acetic acid, and was stored in the dark at room temperature. Stain was prepared by diluting the stock solution 40-fold in water.

PM-Enriched Vesicles

PM vesicles were isolated from roots grown with and without 40 mg L^{-1} HA using differential centrifugation as described by De Michelis and Spanswick (1986), with some modifications (Façanha and de Meis, 1995). In brief, about 15 g (fresh weight) of maize roots was homogenized using a mortar and pestle in 30 mL of ice-cold buffer containing 250 mM Suc, 10% (w/v) glycerol, 0.5% (w/v) polyvinylpyrrolidone-40 (40 kD), 2 mM EDTA, 0.5% (w/v) bovine serum albumin, and 0.1 M Tris-HCl buffer, pH 8.0. Just before use, 150 mM KCl, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride 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, and 1 mM EDTA. The suspension containing membrane vesicles was layered over a 20%/30%/42% (w/w) discontinuous Suc gradient that contained, in addition to Suc, 10 mM Tris-HCl buffer (pH 7.6), 1 mM DTT, and 1 mM EDTA. After centrifugation at 100,000*g* for 3 h in a swinging bucket, the vesicles which sedimented at the interface between 30% and 42% (w/w) Suc were collected, diluted with 3 volumes of ice-cold water, and centrifuged at 100,000*g* for 40 min. The pellet was resuspended in a buffer containing 10 mM Tris-HCl (pH 7.6), 10% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA. The vesicles were either used immediately or frozen under liquid N_2 and stored at −70°C until use. Protein concentrations were determined by the method of Lowry et al. (1951).

ATPase Activity

ATPase activity in PM vesicles was determined by measuring the release of P_i colorimetrically (Fiske and Subbarow, 1925). Between 80% and 95% of the PM vesicles' ATPase activity measured at pH 6.5 was inhibited by vanadate (0.1 mM), a very effective inhibitor of the PM P-type H^+-ATPase (Sze, 1985). In all experiments, the ATPase activity was measured at 30°C, with and without vanadate, and the difference between these two activities was attributed to the PM H^+-ATPase.

ATPase H^+ Pumping

The electrochemical H^+ gradient generated by the H^+-ATPase was estimated from the initial rate of quenching of the fluorescent pH probe ACMA (2 μM, 415-/485-nm excitation/emission), and expressed in percentage quenching per minute. The assay medium contained 10 mM HEPES-KOH (pH 6.5), 100 mM KCl, 3 mM $MgCl_2$, 2.5 μM ACMA, and 0.05 mg L^{-1} PM vesicles protein. The reaction was triggered by addition of 1 mM ATP.

Western Blot

PM vesicles isolated from maize roots, treated or not with HAs, were incubated at 65°C for 10 min, separated on 7.5% (w/v) SDS-PAGE, and transferred to a nitrocellulose membrane. After blocking (5% [w/v] dry milk in phosphate-buffered saline), the nitrocellulose membrane was probed with anti-H^+-ATPase PMA2 (from *Nicotiana plumbaginifolia* Viv.) polyclonal antibodies. These PMA2 antibodies recognized all the isoforms of the enzyme (Morsomme et al., 1996) at a dilution of 1:5,000. The detection of the H^+-ATPase was carried out with the rabbit peroxidase-linked secondary antibodies revealed using diamino-benzidine tetra-hydrochloride. The spots on the nitrocellulose membrane were quantified densitometrically as described by Retamal et al. (1999).

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