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Alkamides: a new class of plant growth regulators linked to humic acid bioactivity

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Abstract

Background: The use of humic substances as plant biostimulants has been increasingly attracting farmers and stunning researchers. The ability of these substances to enhance root growth by changing root architecture is often linked to their hormonal activities, such as auxin effects and nitric oxide production. Humeomics accesses the molecular constituents of humic substances, revealing the importance of alkyl components because of their conformations and chemical activities. Here, we describe the alkamides present in humic acids and compare their bioactivities using plasma membrane H^+-ATPase activity level as a biochemical marker.

Methods: Humic acids isolated from vermicompost were analyzed using ^{13}C and ^{15}N nuclear magnetic resonance spectroscopy. The unbound fraction was extracted with ethyl acetate and submitted to gas chromatography coupled to mass spectrometry to detect the presence of *N*-isopropyldecanamide. We synthesized *N*-isopropyldecanamide and treated maize seedlings for 7 and 15 days with different concentrations. The root growth and plasma membrane H^+-ATPase activity were monitored. Nitric oxide accumulation in the lateral roots was imaged using 4,5-diaminofluorescein diacetate. The results were compared with those obtained for seedlings treated with humic acids isolated from vermicompost.

Results: The amide functional group produced the only nitrogen signal in the ^{15}N humic acid resonance spectrum and similar alkamide moieties were found in the unbound humic extract through comparisons using gas chromatography coupled to mass spectrometry. The synthesis of *N*-isopropyldecanamide had few steps and produced a high yield (86%). The effects of *N*-isopropyldecanamide on root growth were concentration dependent. High concentrations (10^{-4} M) enhanced root growth after 15 day of diminishing shoot biomass. However, low concentrations (10^{-8} M and 10^{-6} M) promoted root growth at 7 and 15 days, similar to the humic acid-induced plasma membrane H^+-ATPase activity. Both *N*-isopropyldecanamide and humic acids enhanced nitric oxide accumulation during lateral root emergence.

Conclusion: We described for the first time the effects of *N*-isopropyldecanamide on the plasma membrane H^+-ATPase activity in maize seedling roots and compared its effects with those caused by humic acids. *N*-Isopropyldecanamide was detected in the unbound fraction of the humic supramolecular assembly, indicating that the putative hormone-like effects of these substances result also from the presence of this new class of plant regulators, in addition to other molecules.

Keywords: Affinins, Small lipids, Hormone-like effects, Plant growth regulators, Humic substances

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Background

Humic substances (HS), constituting a category of plant biostimulants, can be used directly on plants in low concentration to enhance nutrient uptake, plant growth and yield [1]. The effects of HS on plant physiology and metabolism have been attributed to their putative hormone activities [2], which are mainly auxin-like [3–6] because other plant hormones, such as gibberellins, cytokinins, nitric oxide (NO) and ethylene, are found in insignificant concentrations in soils and HSs [7–14]. Curiously, the main nitrogen species often related to humic structures by ^{15}N nuclear magnetic resonance (NMR) spectroscopy are present in amide functional groups, as revealed by the dominant peaks between −245 and −260 ppm [15, 16]. Furthermore, the recalcitrant or hydrophobic nature of HS was previously related to their bioactivities [17–20].

In the early 2000s, a new class of plant growth regulators, called affinins, was described by Ramirez-Chávez et al. [21]. Alkamides are secondary metabolites comprising over 200 related compounds having a general structure that originates from the condensation of an unsaturated fatty acid and an amine. Alkamides promote lateral root formation and root hair elongation, which are similar to the effects produced by auxins, but the ability of the root system to respond to affinins is independent of auxin signaling [21]. The mechanisms through which the alkamides affect particular signal transduction cascades that modify root growth and differentiation are unknown, but the involvement of cytokinin receptors and NO production have been reported during root development [22, 23]. In addition, Morquecho-Contreras et al. [23] related the structures of alkamides, such as *N*-isopropyldecanamide, to the bacterial quorum-sensing signals, *N*-acyl-L-homoserine lactones. These compounds participate in cell-to-cell signaling, usually referred to as quorum sensing, which is a fundamental step in endophytic bacteria biofilm formation and host colonization [24]. Considering the effects of biostimulants manufactured using humic acids (HA) and plant growth-promoting bacteria on plant physiology [25–27], as well as the wide distribution of these lipids in unbound soil fractions and compost humeomes [28–32], we determined whether (i) this class of compounds was linked to HA bioactivity levels and whether (ii) alkamides are present in the supramolecular structure of HA.

HA change the cellular electrical environment by enhancing H^+ efflux [33]. Proton pump activity levels can be used as biochemical markers of HA bioactivity [34]. We synthesized *N*-isopropyldecanamide, an abundant plant alkamide, and used different concentrations to treat maize seedlings. The root growth and the number of root mitotic sites were measured, as well as the effects on the plasma membrane H^+-ATPase activity level and NO production. The unbound HA fraction from cattle manure vermicompost was obtained using an organic solvent, and *N*-isopropyldecanamide was isolated from the humic supramolecular assembly. The alkamide structures in the HA were identified using a retention time comparison and mass fragmentation analysis.

Materials and methods

Synthesis of *N*-isopropyldecanamide

A solution of 29.0-mmol decanoic acid (5.0 g) in 54.8-mmol thionyl chloride (4.0 mL) was stirred and heated to reflux for 12 h. The excess thionyl chloride was removed by distillation, and the residue containing the decanoyl chloride was used in the next step without purification. A solution of 58.7-mmol isopropylamine (5 mL) in hexane (10 mL) was added dropwise to a stirred solution of decanoyl chloride in hexane (20 mL). During this period, the temperature of the reaction was maintained at 0–5 °C. Afterward, it was stirred at room temperature for 5 h. The salts were removed by filtration and washed twice with 20 mL H_2O. The organic layer was dried and evaporated in a vacuum, and the resulting solid was purified by recrystallization from ethyl ether to yield *N*-isopropyldecanamide as a white solid (5.3 g, 86% yield) with m.p at 47 °C. The characterization of *N*-isopropyldecanamide was performed using NMR and gas chromatography coupled with mass spectroscopy (GC–MS) experiments. The ^1H and ^{13}C NMR spectra were recorded on a Jeol 400 instrument (^1H: 400 MHz and ^{13}C: 100 MHz; Tokyo, Japan) with TMS as the internal standard. Electron ionization (EI) mass spectra were obtained using a GC–MS Shimadzu QP5050A instrument at 70 eV. A DB-5 capillary column (30 m, 0.25 nm i.d.) was used with a heating rate of 15 °C min^{-1} from 50 to 230 °C. The injector temperature was set at 200 °C.

HA extraction and its chemical characterization

The HA used in this study were isolated from the vermicompost of cattle manure. HA were obtained according to the classical method of extraction, isolation and purification described on the Web page of the International Humic Substances Society (www.ihss.gated.edu). After freeze drying by lyophilization, the carbon content was analyzed using dry combustion (CHN analyzer Perkin Elmer series 2400, Norwalk, CT, USA). The chemical nature of the HA was accessed by cross-polarization magic angle spinning (CP/MAS) ^{13}C and ^{15}N NMR. The spectrum was acquired from the solid sample using a Bruker Avance 300 MHz (Bruker, Karlsruhe, Germany) equipped with a 4-mm wide bore MAS probe operating at a ^{13}C-resonating frequency of 75.47 MHz. The ^{13}C spectrum was integrated over the chemical shift

(ppm) resonance intervals of 0–46 ppm (alkyl C, mainly CH_2 and CH_3 sp^3 carbons), 46–65 ppm (methoxy and N alkyl C from OCH_3, C–N and complex aliphatic carbons), 65–90 ppm (*O*-alkyl C, such as alcohols and ethers), 90–108 ppm (anomeric carbons in carbohydrate-like structures), 108–145 ppm (phenolic carbons), 145–160 ppm (aromatic and olefinic sp^2 carbons), 160–185 ppm (carboxyl, amides and esters) and 185–225 ppm (carbonyls). The unbound fraction associated with HA was extracted from 100 mg of sample suspended in 1 mL of ethyl acetate at a pH previously adjusted to 11.0 with 1-M NaOH by stirring for 24 h at room temperature. The supernatant was separated by centrifugation (15 min, 3500×*g*), and the aliquot was injected into a Shimadzu QP5050A GC–MS (Tokyo, Japan) at 70 eV using a DB-5 capillary column (30 m; 0.25 nm d.i.) at 15 °C min^{-1} from 50 to 230 °C. The sample was injected at 200 °C.

Plant growth and HA treatment

Maize seeds (*Zea mays* L., var UENF 506) were surface sterilized by soaking in 0.5% NaClO for 30 min, rinsed and then soaked in water for 6 h. Afterward, the seeds were sown on wet filter paper and germinated in the dark at 28 °C. In the first experiment, 4-day-old maize seedlings with ~1 cm roots were transferred into a solution containing 2 mM $CaCl_2$ with or without 20 mg C_{AH} L^{-1} extracted from earthworm compost or 10^{-4}, 10^{-6} or 10^{-8} M *N*-isopropyldecanamide. A minimal medium (2 mM $CaCl_2$) was used to avoid any interference by nutrient constituents that could act synergistically with HA on plant growth and metabolism. In the second experiment, 4-day-old maize seedlings were transferred to Leonard pots containing sterile sand. On the first day, 500 mL half-strength Hoagland's solution plus 20 mg C of HA or 10^{-4}, 10^{-6} or 10^{-8} M *N*-isopropyldecanamide was added. The nutrient solution without HA or *N*-isopropyldecanamide was changed weekly. The roots were collected from 7- to 15-day-old seedlings in the first and second assays, respectively.

Root growth measurements

Root lengths and areas were measured using a Delta-T Scan software image analyzer (Delta-T Devices, Ltd, Cambridge, England). Other samples of root seedlings were collected and used in additional experiments.

Frequency of sites of lateral root emergence

The entire root systems were washed in water and cleaned by boiling at 75 °C for 20 min in 0.5% KOH. Afterward, root samples were rinsed in water and stained with a hematoxylin solution for 14 h in the dark. They were then rinsed in water and destained in 80% lactic acid at 75 °C for 30 to 90 s. Individual entire roots were transferred to Petri plates containing water and observed with a stereoscopic microscope to evaluate the number of visible mitotic sites on the root tissue. The hematoxylin stock solution contained 1 g hematoxylin, 0.5 g ferric ammonium sulfate and 50 mL 45% acetic acid, and it was stored in the dark at room temperature. Stains were prepared by diluting the stock solution 40-fold in water.

Plasma membrane (PM)-enriched vesicles

The PM-enriched vesicles were isolated from roots using differential centrifugation. Briefly, ~15 g (fresh weight) of maize roots was homogenized using a mortar and pestle in 30 mL of ice-cold buffer containing 250-mM sucrose, 10% (w/v) glycerol, 0.5% (w/v) PVP (40 kDa), 2-mM EDTA, 0.5% (w/v) BSA and 0.1-M Tris–HCl buffer at pH 8.0. Just prior to use, 150-mM KCl, 2-mM DTT and 1-mM PMSF were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at 8000×*g* for 10 min. The supernatant was centrifuged once again at 8000×*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 PM vesicles was layered over a 20%/30%/42% (w/w/w) discontinuous sucrose gradient that contained, in addition to sucrose, 10-mM Tris–HCl (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 at the interface between 30 and 42% sucrose were collected, diluted with three 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 in liquid N_2 and stored at −70 °C until use. Protein concentrations were determined using Lowry's method [35].

Plasma membrane H^+-ATPase hydrolysis

The hydrolytic H^+-ATPase activity levels in the PM-enriched vesicles were determined colorimetrically by measuring the release of P_i [14]. Between 70 and 90% of the PM vesicle's' 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. The assay medium consisted of 1-mM ATP–BTP, 5-mM $MgSO_4$, 10-mM MOPS–BTP (pH 6.5), 100-mM KCl, 0.2-mM Na_2MoO_4 and 0.05 mg mL^{-1} vesicle protein. In the experiments, ATPase activity was measured at 30 °C,

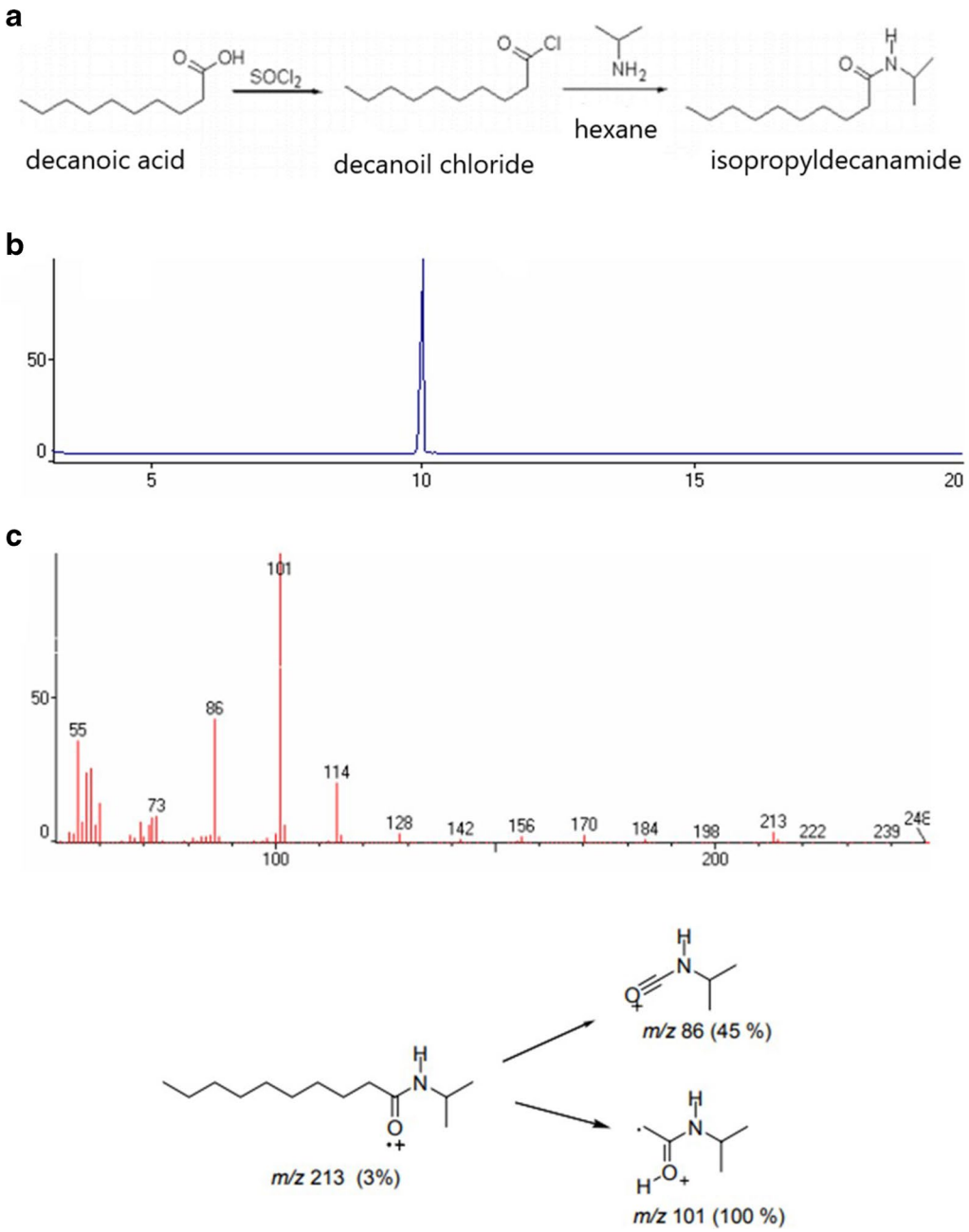


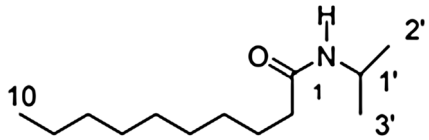
Fig. 1 **a** Synthesis pathway of *N*-isopropyldecanamide; **b** gas chromatography and **c** mass spectroscopy of *N*-isopropyldecanamide fragmentation

with and without vanadate, and the difference between the two measurements was attributed to the PM H^+-ATPase.

H^+-pumping by PM H^+-ATPase

The electrochemical H^+-gradient generated by the H^+-ATPase was estimated from the initial quenching rate of the fluorescent pH probe 9-amino-6-chloro-2-methoxyacridine (415/485 nm excitation/emission) and expressed in percentage quenching per min. The assay medium contained 10-mM HEPES–KOH (pH 6.5), 100-mM KCl, 3-mM $MgCl_2$, 2.5-μM 9-amino-6-chloro-2-methoxyacridine and 0.05 mg L^{-1} PM vesicles protein. The reaction was triggered by the addition of 1-mM ATP. The addition of either 3-μM FCCP or 2-μM NH_4Cl abolished the H^+ gradient created by ATP hydrolysis.

Table 1 *N*-Isopropyldecanamide ^{13}C NMR (100 MHz) and ^{1}H (400 MHz) H chemical shift (*δ*) spectral



Position	δ_C (ppm)	δ_H (ppm)
1	172.21	
2	36.98	2.12 t (*J* = 7.4)
3	25.79	1.61 m
4	29.22	1.28 m
5	29.22	1.28 m
6	29.39	1.28 m
7	29.31	1.28 m
8	31.79	1.28 m
9	22.60	1.28 m
10	14.02	0.88 t (*J* = 7.0)
1′	41.09	4.09 sp (*J* = 6.6)
2′ and 3′	22.77	1.4 d (*J* = 6.6)

Fig. 3 Maize root seedlings independently treated with 20 mg C L^{-1} humic acids (HA) and 10^{-4}, 10^{-6} and 10^{-8} M *N*-isopropyldecanamide collected at 7 and 15 days

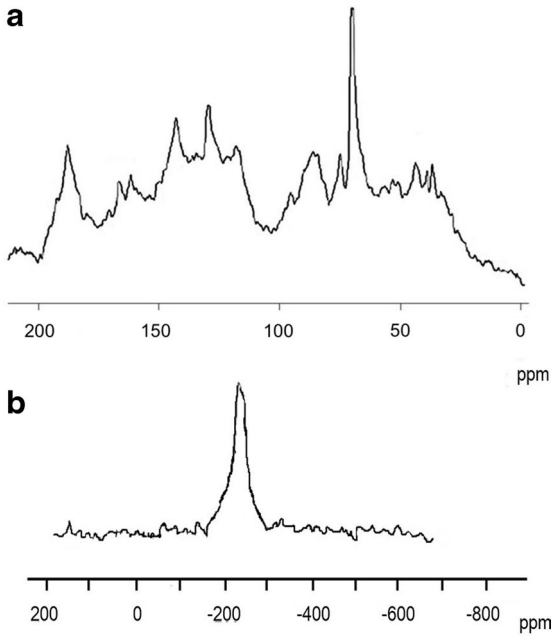


Fig. 2 Cross-polarization and magic angle spinning of ^{13}C and ^{15}N nuclear magnetic resonance of humic acids isolated from cattle manure vermicompost

NO measurement and localization

The NO was imaged using 4,5-diaminofluorescein diacetate (DAF-2 DA) with a fluorescence microscope. Root transverse sections from mature zones treated for 72 h were loaded with 10-μM DAF-2 DA in 10-mM HEPES-BTP buffer (pH 7.5) for 40 min, washed three times in fresh buffer and analyzed microscopically (488 nm/495–575 nm excitation/emission). The transverse root sections were ~5 μm and were created using a table microtome (LPC model, Rolemberg e Bhering Trading and Import, Belo Horizonte, Brazil). Images acquired from the light microscope (Zeiss Axioplan coupled with a Canon A640 digital camera) were analyzed using ImageJ software in the LR zone (~30 mm from the root–seed junction). Maize roots without DAF-2 DA addition were used as blank controls. The same camera settings were used, and the digital images were not processed further. The effects of the NO donor sodium nitroprusside (SNP, 200 μM) and the specific NO scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, 200 μM) on NO production were investigated. At least three samples were measured per treatment in three independent experiments.

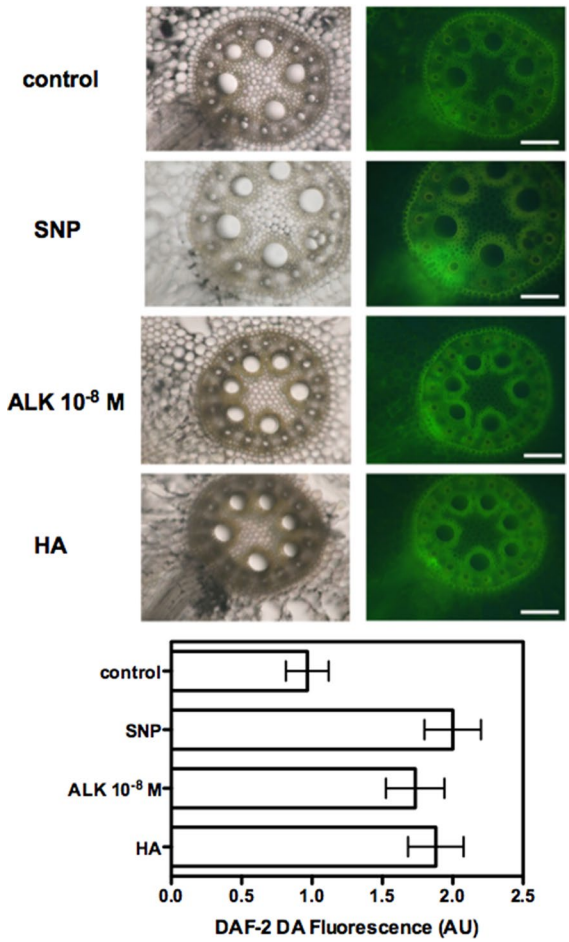


Fig. 4 Nitric oxide production at the site of lateral root emergence. Maize transverse root sections loaded with DAF-2 DA and observed by bright-field and fluorescence microscopy as described in "Materials and methods". Effects of independent 20 mg C L^{-1} humic acids (HA), 10^{-8} M *N*-isopropyldecanamide and 200 µM sodium nitroprusside (SNP) treatments. The fluorescence intensity of the images was estimated by measuring the pixel intensity and analyzed with ImageJ (NIH) software. Data are expressed as arbitrary units (AUs). Means ± SDs of three independent experiments performed in triplicate are shown. Scale bars = 50 µm

Results

Alkamide synthesis

N-Isopropyldecanamide was synthesized from decanoic acid by the acylation of isopropylamine with decanoyl chloride prepared in situ (Fig. 1a), resulting in an 86% yield. The *N*-isopropyldecanamide's structure was confirmed by spectral data involving ^{1}H, ^{13}C NMR and GC–EIMS (Table 1). The EI mass spectrum of *N*-isopropyldecanamide (Fig. 1b, c) has a molecular peak at *m/z* 213 ($[M]^{+}$). The observed peaks at *m/z* 86 (45%) and 101 (100%) are in agreement with the presence of an amide moiety (Fig. 1b). The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (Table 1) were compatible with the structure of *N*-isopropyldecanamide.

HA characterization by CP–MAS ^{13}C and ^{15}N NMR

The CP/MAS ^{13}C NMR analysis (Fig. 2a) showed low signals in the methyl and methylene group regions of long alkyl chains (0–40 ppm). A broad peak was observed in the region between 44 and 50 ppm owing to the C bonded to mono- and di-O. The signals at 57 ppm are normally attributed to OCH_3 groups from lignins, and the signal near 65 can be attributed to carbinolic Cs of primary alcohols and polysaccharides. The sharp and well-resolved signal at 71 indicates sp^3 C atoms bound to N. The signals near 100 ppm indicate the presence of anomeric carbons from carbohydrates. The peak centered at 130 ppm is from unsubstituted aromatic carbons. The peaks between 150 and 160 ppm indicate phenolic OH groups. Signals in the region from 160 to 190 ppm indicate the presence of differently substituted carbonyl-C atoms and amides. Quantitatively, the spectra revealed 9% carboxyl, 5% phenolic, 36% aromatic, 32% peptide and carbohydrate, and 18% other aliphatic carbons. The ^{15}N spectrum of the solid state (Fig. 2b) revealed the presence of one broad signal centered at −245 ppm, which is typically attributed to amide groups [16]. The elemental composition of the HA was 48%, 3.8%, 5.0% and 43.2% total carbon, nitrogen, hydrogen and oxygen, respectively. The ash content was low (< 0.5%).

Root dry mass, length and surface area

$CaCl_2$ medium containing different *N*-isopropyldecanamide stimulated root biomass production (Fig. 5). Treatments with 10^{-4} M *N*-isopropyldecanamide did not stimulate root development, although no inhibitory effect on maize growth was observed (Fig. 5). Maize seedlings treated with 20 mg C L^{-1} HA showed strongly stimulated root development (Fig. 5). After 15 days of growth in sand pots, 10^{-4} M *N*-isopropyldecanamide treatments had no effects on root growth when compared with control plants. However, at lower concentrations (10^{-6} and 10^{-8} M), *N*-isopropyldecanamide enhanced root development, producing increases in root dry mass, total length and superficial area (Fig. 5). The use of HA at a 20 mg C L^{-1} concentration also stimulated root development in maize (Figs. 3 and 5).

Root mitotic sites and lateral root emergence

The proliferation of mitotic sites in the root meristematic zones of 7- and 15-day-old plants treated with HA or *N*-isopropyldecanamide are shown in Fig. 6. The lower *N*-isopropyldecanamide (10^{-8} M) treatment promoted an increase in the number of mitotic sites and lateral root emergence, which were very similar to the effects of HA.

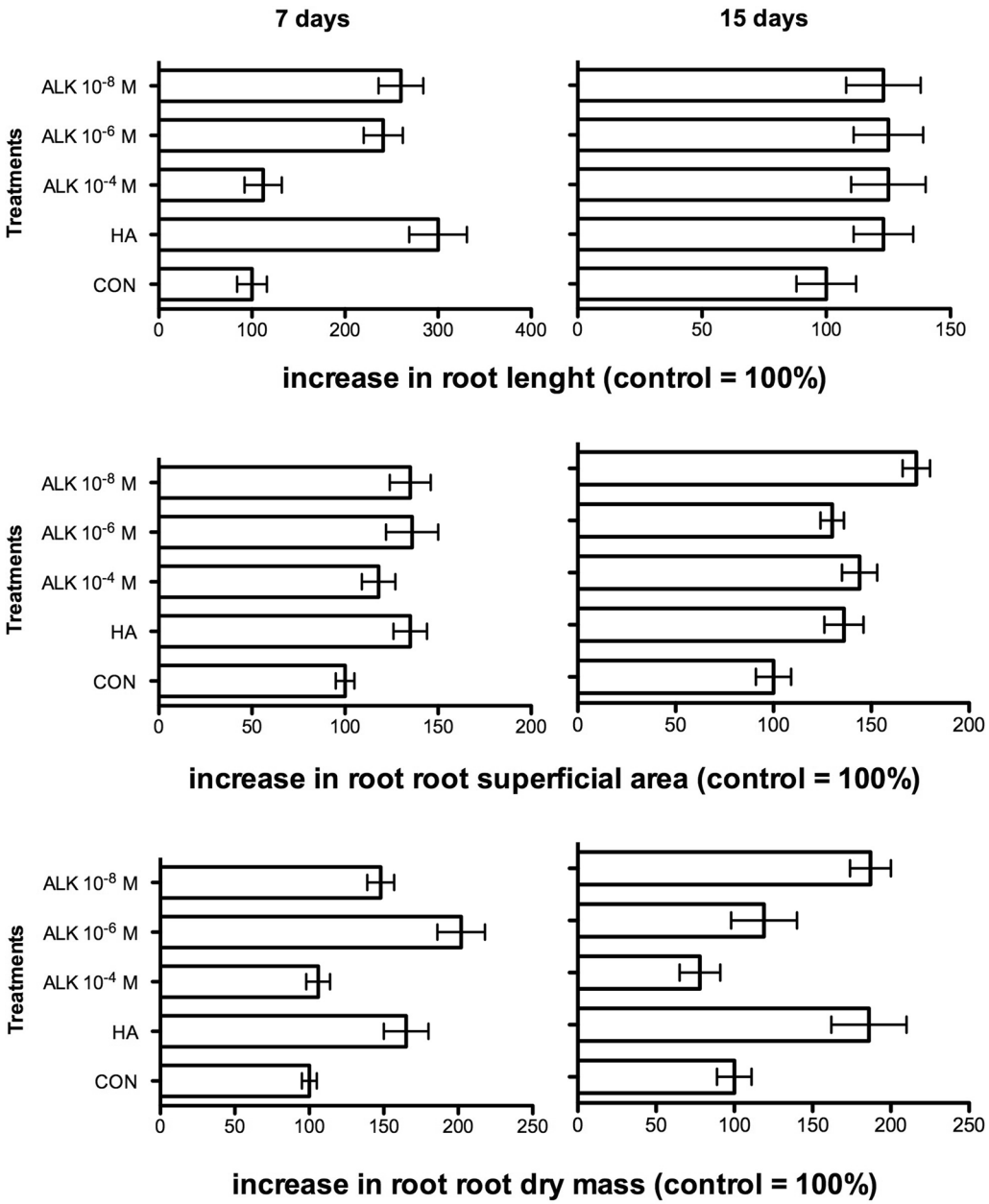


Fig. 5 Effects of independent 10^{-4}, 10^{-6} and 10^{-8} M *N*-isopropyldecanamide and 20 mg C L^{-1} humic acid (HA) treatments on root dry mass, radicular superficial area and length of maize seedlings as analyzed by Delta-T scan software. Total root length was calculated as the sum of the lengths of primary and lateral roots. Data are expressed as percentage of control and represent normalized means from six independent experiments performed with maize seedlings (10 plants per treatment in each case). Bars represent standard deviation values

The stimulative effects observed at 7 days were maintained at 15 days.

H^+ pumping and ATP hydrolysis

PM vesicles isolated from maize roots treated for 7 day with 20 mg C L^{-1} HA or 10^{-4}, 10^{-6} or 10^{-8} M *N*-isopropyldecanamide exhibited clear vanadate-sensitive stimulative effects (Fig. 7a) on the ATP-dependent proton gradient's formation and ATPase activity (Fig. 7b). Both the initial rate of gradient formation and ATP hydrolysis were enhanced by fourfold and threefold in response to HA and *N*-isopropyldecanamide (10^{-6} and 10^{-8} M) treatments, respectively. Thus, the H^+ pump may be involved

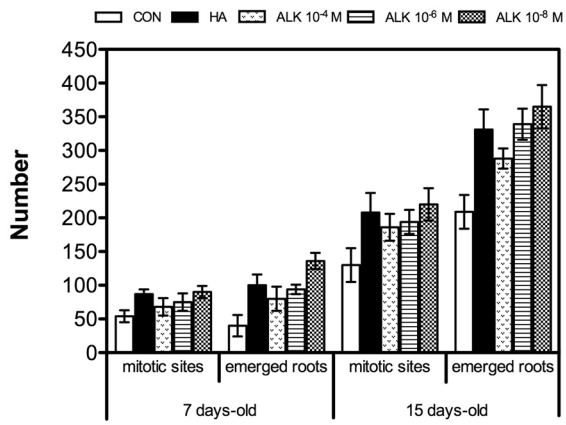


Fig. 6 Effects of independent 10^{-4}, 10^{-6} and 10^{-8} M *N*-isopropyldecanamide and 20 mg C L^{-1} humic acid (HA) treatments on the root growth pattern as evaluated by the quantification of lateral root mitotic sites

in the alkamide-related stimulation of root growth, in a manner similar to that previously observed for HA.

N-Isopropyldecanamide, HA and SNP induced NO accumulation in maize root

The NO fluorescence detected in situ at the mature root zone using the fluorescent probe DAF-2 DA during lateral root formation was enhanced ~100%, 90% and 70% by SNP, HA and *N*-isopropyldecanamide, respectively (Fig. 4). The presence of PTIO reduced the endogenous NO fluorescence by ~50% and reduced the signals obtained after treatments to levels similar to those of untreated seedlings.

Discussion

Alkamides contain an acyl chain linked by an amide bond to an amine-containing head group. The nature of the alkyl amine group may vary, with butyl, isobutyl and propyl groups having been reported. The best studied alkamide is *N*-isobutyldecatrienamine, also named affinin [21]. Here, we synthesized *N*-isopropyldecanamide and observed the presence of decanamide in the unbound fraction associated with HA aggregation (Figs. 1 and 8). The presence of small lipids in the humic fraction had been previously revealed through humeomics, the sequential chemical fractionation of humic matter from different sources [28–32] as well as their chemical conformations [36] and activities [37]. However, here, for the first time, the bioactivities of HA were linked to the presence of alkamides. HA affect nutrient uptake through the synthesis and functionality of membrane proteins, especially proton pumps that increase the electrochemical proton gradient across the PM [38]. Owing to their crucial roles in ion uptake and root growth, they can be used as biochemical markers of HA bioactivity [18]. While the effects of alkamides on root growth are known [21, 22], their effects on PM H^+-ATPase were not considered in previous reports.

In plant cell metabolism, PM H^+-ATPase plays a central role owing to the electrochemical gradient generated by ATP hydrolysis. In this reaction, 3–5 mol of H^+ are produced, which drive the PM electrochemical potential. The energy produced can be used to improve plant nutrition by increasing the electrochemical proton gradient that drives ion transport across the cell membrane through the secondary transport systems [38]. The apoplast acidification loosens the cell wall, allowing cell elongation [39, 40]. The elongation-related differentiation zone of the root includes small, dense meristematic cells that are continuously metabolically active and are more susceptible to lateral root formation. The proliferation of root hairs can dramatically increase the root surface area. Root length and surface area changes (Fig. 5) are important because increases in these parameters are reflective of an increase in the root's absorptive area. In addition, these meristematic zones are differentiation sites and precursors of lateral roots (Fig. 6), and they are formed by cells that have a PM enriched with H^+-ATPases [40]. Therefore, it is possible that an enhanced PM H^+-ATPase activity (Fig. 7) might be associated with the induction of mitotic sites by HA (Fig. 6). Auxin can induce the de novo synthesis of PM H^+-ATPase in plant tissues [39], which is correlated with the induced expression of the major isoform of H^+-ATPase mRNA (Mha2) in maize [40]. Canellas et al. [4] showed an increase in the PM H^+-ATPase content, measured by western blot analysis using antibodies raised against the PMA2 isoform from *Nicotiana plumbaginifolia*, in roots of maize plants exposed to earthworm compost for 7 days. The authors hypothesized that this increase in the Mha2 isoform could result from effects on Mha2 transcription and, considering that the *Mha2* gene's most significant regulatory feature was a threefold increase in its steady-state mRNA level in response to auxin [40], concluded that the actions of HSs on PM H^+-ATPase may rely on the auxin-dependent activation of the *Mha2* gene. The results presented by Quaggitotti et al. [41] confirmed that hypothesis, showing a stimulation of Mha2 mRNA synthesis exclusively at the root level at 48 h after treatment with low-molecular weight HSs. Thus, in HA- and *N*-isopropyldecanamide-treated plants, a significant increase in both hydrolytic activity and the proton transport of PM H^+-ATPase occurs. In addition to the transcriptional regulation, both post-transcriptional and post-translational mechanisms could be involved in controlling enzymatic activities.

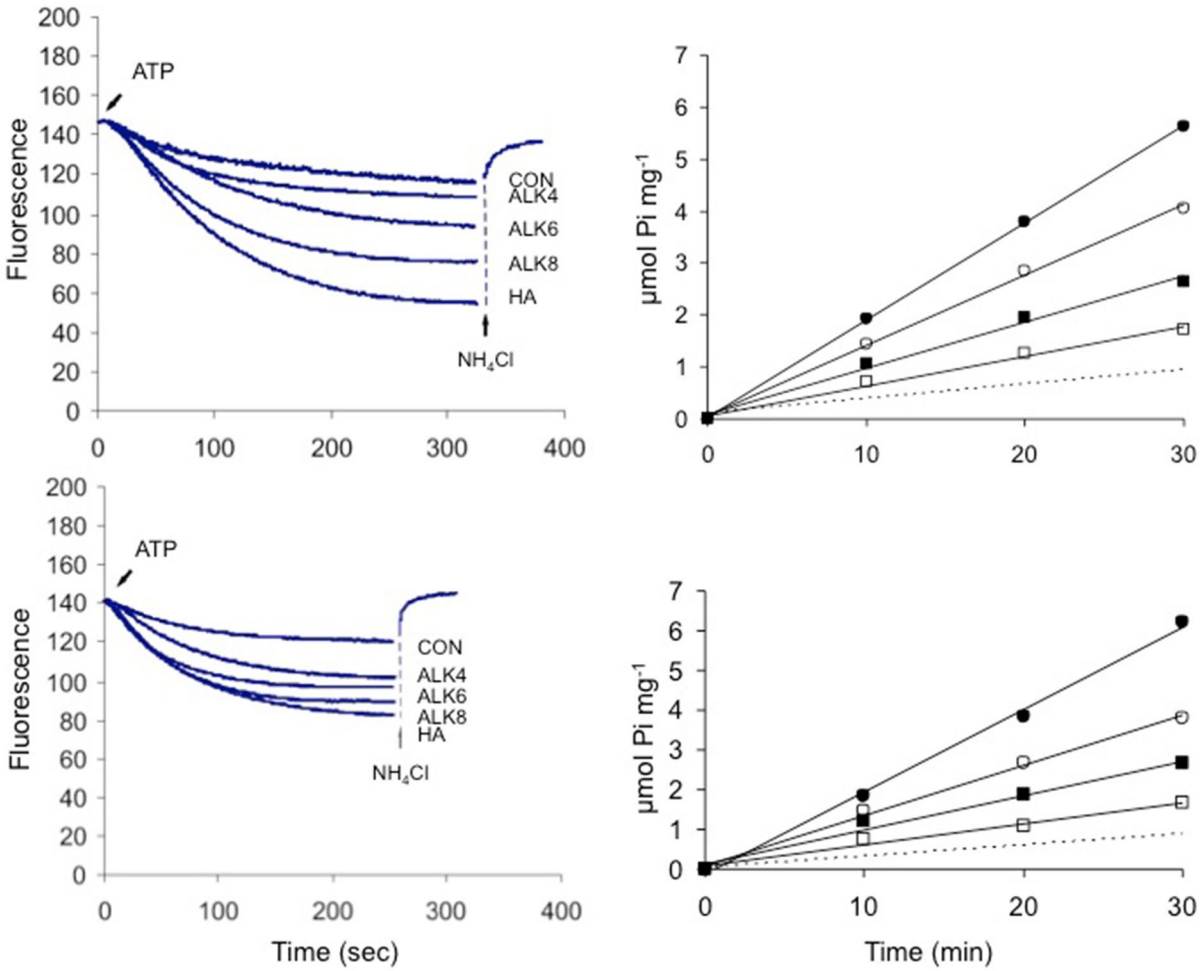


Fig. 7 Effects of different *N*-isopropyldecanamide and humic acid concentrations on plasma membrane H^+-ATPase activity and proton pumping in vesicles isolated from 7-day-old maize seedlings. Vanadate-sensitive ATP hydrolysis and proton transport were measured in plasma membrane vesicles isolated from maize roots treated with 10^{-8} M (white circle), 10^{-6} M (black square) and 10^{-4} M (white square) *N*-isopropyldecanamide and 20 mg C L^{-1} humic acids (black circle) for 7 (**a**) and 15 (**b**) days. Control (dotted line). The reaction medium contained 50-mM HEPES–KOH (pH 6.5), 3-mM $MgSO_4$, 100-mM KCl and 1-mM ATP. **a**, **b** Representative experiments of at least four independent preparations of plasma membrane from maize roots

The phytohormone auxin is a key regulator of lateral root development and root hair formation. Auxin is active over a very wide range of concentrations: low auxin concentrations (10^{-10}–10^{-9} M) stimulate primary root growth, whereas higher concentrations (10^{-8}–10^{-6} M) inhibit primary root growth and stimulate lateral root and root hair formation [5]. An important difference between the HS and auxin modes of action is that auxins induce lateral root formation at concentrations that have an inhibitory effect on primary root growth, while HA can induce lateral root formation at concentrations that enhance primary root growth [42]. Thus, alkamides could alter root growth by a mechanism different from that of auxins. Ramirez-Chávez et al. [21] found that alkamides can stimulate lateral root formation at high concentrations. Further plant treatments with 10^{-8}-M synthetic auxin (2,4-D) induced *DR5:uidA* and *BA3:uidA* expression, whereas concentrations of up to 10^{-4}-M alkamide failed to affect these auxin-inducible gene markers, indicating that the affinin-associated root induction mechanism is different from that of auxin. However, the stimulation of PM H^+-ATPase observed in plants treated with different *N*-isopropyldecanamide concentrations (10^{-4} to 10^{-8} M) suggested the involvement of a new class of plant growth regulators in the energy metabolism and in cellular signaling cascades controlled by electrogenic pumps. The presence of alkamides in the colloidal dispersion of HA (Fig. 8) suggests that HSs can act as

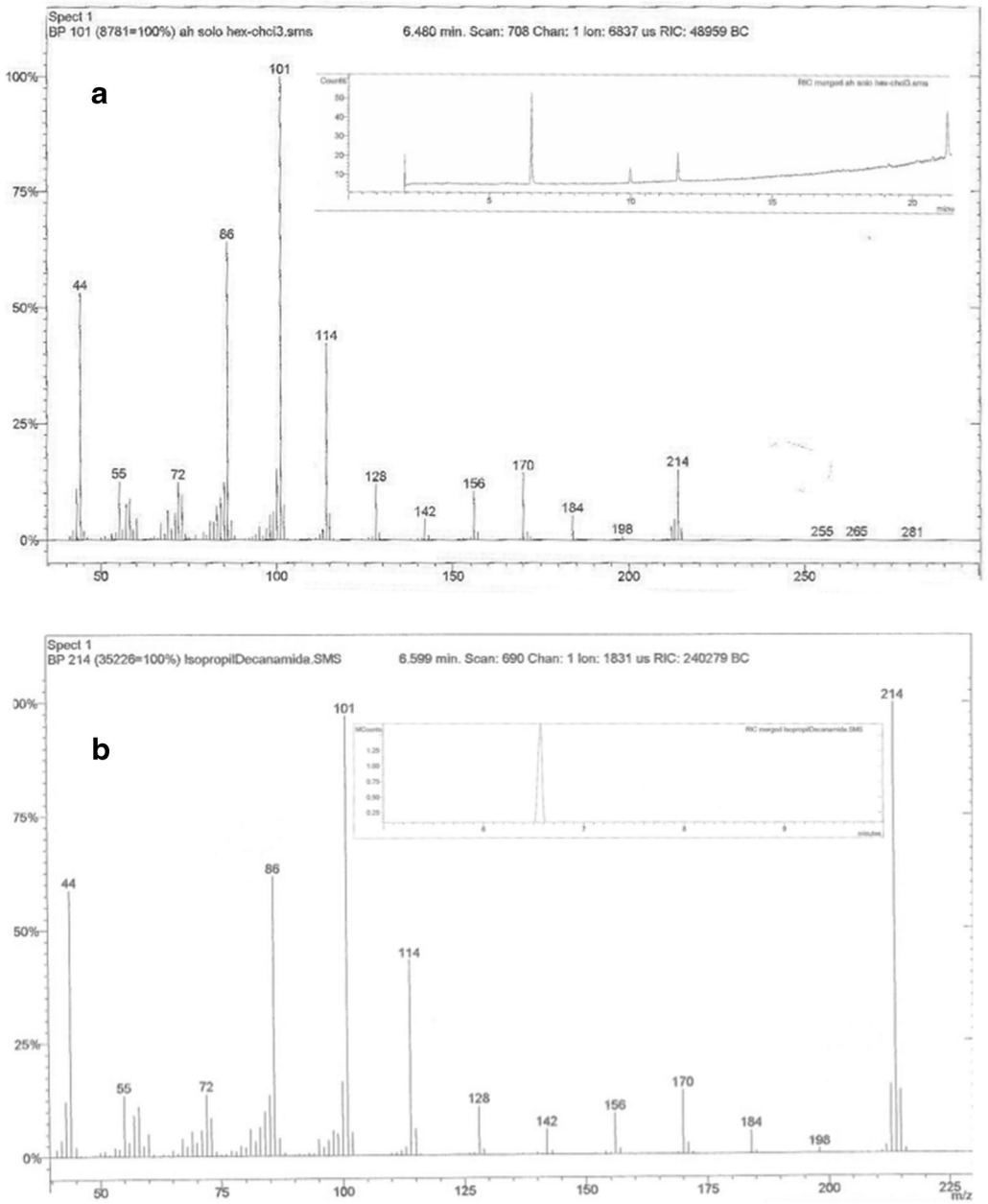


Fig. 8 Gas chromatography and mass spectroscopy of *N*-isopropyldecanamide isolated from a humic acid structure with ethyl acetate

sources of several chemical groups with high biological activities.

The most common effects of HS on plant development are related to hormonal and the auxin-like activities [2]. However, a new group of plant growth-regulating substances has an apparently auxin signaling-independent response [21]. The influence of HS on different enzymes has been demonstrated [43]. Here, the clear stimulation of root development by in vivo *N*-isopropyldecanamide and HA treatments was shown and correlated with an enhanced PM H^+-ATPase activity in 7- and 15-day-old plants. Since auxin inhibitors could only partially impair HA bioactivity [14], it seems that the remaining HA effects could be related to alkamides. The 10^{-6}- and 10^{-8}-M *N*-isopropyldecanamide treatments, which enhanced root length and superficial area significantly, positively altered the PM H^+-ATPase activity, as assessed by two- to threefold increases in ATP hydrolysis and

ATP-dependent H^+ transport compared with control plants. Root growth promotion by HA has been reported and can be explained, at least in part, by an enhancement in PM H^+-ATPase activity. In this work, a 10^{-8}-M *N*-isopropyldecanamide treatment had effects on the initial and steady-state H^+ gradient rates that were very similar to those of HA.

The lateral root formation induced by HA is a well-studied NO-mediated process [14]. The role of NO in the alterations induced by *N*-isobutyldecanamide during lateral root emergence in *Arabidopsis* was studied by Méndez-Bravo et al. [44]. They observed a modulation in auxin-inducible gene expression and lateral root promotion through the interactions of alkamides with signals from jasmonic acid and NO. They concluded that *N*-isobutyldecanamide and its interacting signals with jasmonic acid and NO act downstream or independently of auxin-responsive gene expression to promote lateral root formation [44]. In addition, López-Búcio et al. [22] showed that alkamides may belong to a class of endogenous signaling compounds that interact with the cytokinin-signaling pathway to control meristematic activity and differentiation processes during plant development. Changes in the expression of the cell division marker CycB1:uidA and the enhanced expression of the cytokinin-inducible marker ARR5:uidA occur both in roots and in shoots after plant exposure to alkamides. The presence of alkamides in the HA may contribute to the increased plant cell signaling and accelerated metabolism. The cellular energy balance could be altered as demonstrated by the increase in PM H^+-ATPase activity induced by alkamides and HA.

Conclusion

We describe for the first time the presence of *N*-isopropyldecanamide in the unbound fraction of HA isolated from cattle manure vermicompost. A synthesized alkamide promoted maize root growth in a manner similar to that of HA. In addition, the effects of *N*-isopropyldecanamide on the PM H^+-ATPase activity and NO accumulation in maize roots were shown. In this study, we provide evidence that alkamides enhance PM H^+-ATPase activity and that the bioactivity levels of HA are not only a result of auxin-related effects, but also the presence of a mixture of plant growth regulatory substances.

Abbreviations

ACMA: 9-amino-6-chloro-2-methoxyacridine; ATP: adenosine triphosphate; Tris–HCl: Tris(hydroxymethyl)aminomethane hydrochloride; DTT: DL-dithiothreitol; EDTA: ethylenediaminetetraacetic acid; GC–EIMS: gas chromatography coupled to mass spectrometry with ionization by electron impact; PM H^+-ATPase: plasma membrane proton ATPase; PVP: polyvinylpyrrolidone; PMSF: phenylmethanesulfonyl fluoride; HA: humic acids isolated from cattle manure vermicompost; SNP: sodium nitroprusside; PTIO: 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; DAF-2 DA: 4,5-diaminofluorescein diacetate; NO: nitric oxide.

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Authors' contributions

DBZ carried out the plant experiments; CM wrote the first version of this paper; CRRM synthesized the *N*-isopropyldecanamide and confirmed its structure by spectroscopy methods; RNC found the *N*-isopropyldecanamide in the supramolecular arrangement of humic acids; RS did the humic acid characterization using CP/MAS NMR; LPC conceived the experiment and wrote the final version. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Ethics approval and consent to participate

This manuscript is an original paper and has not been published in other journals. The authors agreed to follow the copyright rule.

Consent for publication

The authors agreed to the publication of the manuscript in this journal.

Competing interests

The authors declare that they have no competing interests.

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