



# Root ABA and H<sup>+</sup>-ATPase are key players in the root and shoot growth-promoting action of humic acids

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## Funding information

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## Abstract

Although the ability of humic (HA) and fulvic acids (FA) to improve plant growth has been demonstrated, knowledge about the mechanisms responsible for the direct effects of HA and FA on the promotion of plant growth is scarce and fragmentary. Our study investigated the causal role of both root PM H<sup>+</sup>-ATPase activity and ABA in the SHA-promoting action on both root and shoot growth. The involvement of these processes in the regulation of shoot cytokinin concentration and activity was also studied. Our aim was to integrate such plant responses for providing new insights to the current model on the mode of action of HA for promoting root and shoot growth. Experiments employing specific inhibitors and using *Cucumis sativus* L. plants show that both the root PM H<sup>+</sup>-ATPase activity and root ABA play a crucial role in the root growth-promoting action of SHA. With regard to the HA-promoting effects on shoot growth, two pathways of events triggered by the interaction of SHA with plant roots are essential for the increase in root PM H<sup>+</sup>-ATPase activity—which also mediates an increase in cytokinin concentration and action in the shoot—and the ABA-mediated increase in hydraulic conductivity (L<sub>p</sub>).

## KEYWORDS

abscisic acid, cytokinin, humic acid, humic substances, plant development, root PM H<sup>+</sup>-ATPase

## 1 | INTRODUCTION

Humic substances (HS) are principal components of both soil organic matter (SOM) and dissolved organic matter (DOM) (Canellas & Olivares, 2014; Chen, Nobili, & Aviad, 2004; Olaetxea et al., 2018; Stevenson, 1994; Tipping, 2002; Trevisan et al., 2011). Although the structure of HS is still a matter of debate (Baigorri, Fuentes,

Gonzalez-Gaitano, & García-Mina, 2007a; Baigorri, Fuentes, Gonzalez-Gaitano, & Garcia-Mina, 2007b; Clapp & Hayes, 1999; Piccolo, 2002; Swift, 1989), recent studies indicate that they have singular structural features that are not present in natural, well-characterized, biomolecules, such as proteins, cellulose, lignin as well as microbial metabolites (Cao & Schmidt-Rohr, 2018). These structural features include the distribution of C=O groups in highly substituted

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aromatic blocks and in aliphatic moieties with high degree of chemical bond conjugation, as well as the presence of C-O alkyl chains (Cao & Schmidt-Rohr, 2018).

In solution, HS are organized in complex systems including both individual macromolecules and molecular aggregates (Baigorri, Fuentes, Gonzalez-Gaitano, & García-Mina, 2007a; Baigorri, Fuentes, Gonzalez-Gaitano, & Garcia-Mina, 2007b). Some of these molecules and molecular aggregates self-assemble in solution by combining hydrophilic and hydrophobic moieties (Piccolo, 2002). These special molecular assemblies have new physico-chemical properties (e.g., surfactant features) and may be considered as a new type of natural supramolecules (Piccolo, 2002). Based on their different solubilities in water as a function of pH, HS have been fractionated into three major types: humic acids (soluble at alkaline pH but insoluble at acidic pH) (HA), fulvic acids (soluble at both alkaline and acidic pH) (FA), and humin (insoluble regardless of pH value) (Stevenson, 1994).

Many studies have demonstrated that the presence of HS is a major factor influencing soil fertility (Chen et al., 2004; MacCarthy, Clapp, Malcom, & Bloom, 1990; Monda, Cozzolino, Vinci, Spaccini, & Piccolo, 2017). This action results from the HS capacity to improve plant nutrient availability in soil, mainly through the formation of stable complexes with metals (Chen et al., 2004; Erro et al., 2012; Garcia-Mina, 2006; Gerke, 2010; Urrutia et al., 2014). Besides this effect on improving nutrient bioavailability, HS also promotes plant growth through action on plant metabolism and physiology, which is derived from the interaction of HS with plant roots (Canellas, Olivares, Okorokova-Facanha, & Facanha, 2002; Muscolo, Sidari, Francioso, Tugnoli, & Nardi, 2007; Olaetxea et al., 2015, 2018; Quaggiotti et al., 2004). However, knowledge about the mechanisms responsible for this direct effect of HS on the promotion of plant growth is scarce and fragmentary, regarding the effects on both root development and shoot development (García et al., 2016; Mora, Bacaicoa, Baigorri, Zamarreño, & García-Mina, 2014a; Olaetxea et al., 2018).

With regard to the HA-mediated action on root growth and architecture, a number of studies proposed that it is mainly mediated by auxin signaling pathways (Canellas et al., 2011; Trevisan, Francioso, Quaggiotti, & Nardi, 2010a; Trevisan, Pizzeghello, et al., 2010b; Zandonadi, Canellas, & Façanha, 2007; Zandonadi et al., 2010). In fact, the application of inhibitors of IAA action in plants treated with HA avoided some of the HS-mediated effects on root development, principally those related to root morphology and lateral root proliferation (Zandonadi et al., 2010). Moreover, studies using the DR5::GUS construct (a genetic construct where the synthetic auxin-responsive promoter DR5 controls the expression of bacterial  $\beta$ -D-glucuronidase) (Bierfreund, Reski, & Decker, 2003) in several plant species showed that the application of HA to the roots induced the upregulation of genes controlled by auxin-dependent signaling pathways (Trevisan, Pizzeghello, et al., 2010b). These effects were also linked to NO- and ethylene-dependent signaling pathways (Mora, Baigorri, Bacaicoa, Zamarreño, & García-Mina, 2012; Zandonadi et al., 2010), as well as to significant increases in root plasma membrane (PM) H<sup>+</sup>-ATPase activity and root ABA concentration (Canellas et

al., 2002; Mora, Bacaicoa, et al., 2014a; Mora et al., 2010; Olaetxea et al., 2015; Zandonadi et al., 2010). Some authors have proposed the hypothesis that the HA-mediated increase in root development may result from a coordinated effect on proton and Ca<sup>2+</sup> effluxes associated with root PM H<sup>+</sup>-ATPase activity under the "acid-growth theory" (Ramos et al., 2015). However, there is a lack of mechanistic studies supporting the relevance of this hypothesis.

Therefore, studies oriented to prove the causal role of the HA-induced increase in root PM H<sup>+</sup>-ATPase activity in the whole action of HA on root growth are of great interest.

With regard to the shoot-promoting action of HA, recent studies indicated that it is regulated by the action of HA on IAA-NO signaling pathways in the root (Mora, Bacaicoa, et al., 2014a). Further studies also showed that an increase in root ABA concentration, which is linked to increases in root plasma membrane aquaporin activity and root hydraulic conductivity (L<sub>p</sub>), was crucial for the enhancement in shoot growth caused by HA (Olaetxea et al., 2015). A previous study proposed the hypothesis that the HA capacity to enhance shoot growth involves an increase in cytokinin activity in the shoot which is in turn linked to the HA-mediated increase in the root PM H<sup>+</sup>-ATPase activity and uptake of nitrate by root (Mora et al., 2010). However, as in the case of the mechanisms involved in the action of HA in roots, there is a lack of direct experimental evidence supporting a causal role of both cytokinin activity in leaves and root PM H<sup>+</sup>-ATPase activity in the mechanism responsible for the shoot growth-promoting action of HA. Likewise, the possible functional relationships between the signaling pathways involved in the HA regulation of root PM H<sup>+</sup>-ATPase activity and L<sub>p</sub> remain unknown.

With the aim of investigating in depth the mechanism of action responsible for the enhancement of plant growth caused by HA, the following hypotheses are addressed in the present study:

- (i) The HA-mediated increase in root PM H<sup>+</sup>-ATPase plays an essential role in the capacity of HA to increase root growth.
- (ii) The increase in root PM H<sup>+</sup>-ATPase activity caused by HA plays an essential role in its capacity to enhance shoot growth through an increase in the leaf concentration of cytokinins.
- (iii) The increase in root ABA concentration is also causally involved in the HA-mediated enhancement of root growth.
- (iv) The HA capacity to increase both root PM H<sup>+</sup>-ATPase activity and root ABA concentration is functionally interconnected to each other.

HA with a sedimentary origin and extracted from leonardite (SHA), which has been extensively characterized in previous studies (Aguirre et al., 2009; Mora, Bacaicoa, et al., 2014a; Mora et al., 2010, 2012; Olaetxea et al., 2015), was employed for the experiments. As in previous studies, the plant species used in the experiment was cucumber (Aguirre et al., 2009; Mora, Bacaicoa, et al., 2014a; Mora et al., 2010, 2012; Olaetxea et al., 2015).

It is clear that the whole action of HS on crop development and final yield involves other complementary mechanisms that are not studied here. Indeed, in the case of intensive field crops, those

mechanisms associated with the improvement of nutrient (mainly micronutrient) availability, resulting from the interaction of HS with soil components (Chen et al., 2004; Olaetxea et al., 2018), may be much more relevant than those linked to HS direct action on plant roots. In fact, the main purpose of this research is to investigate some aspects of the action of HS on plant growth as a main component of SOM, which would be directly involved in the role of SOM in soil fertility.

## 2 | MATERIALS AND METHODS

### 2.1 | Extraction and purification of a leonardite humic acid (SHA)

The SHA sample was obtained from leonardite (Danube basin). Previous studies extensively described the IHSS methodology (<http://www.humicsubstances.org/soilhafa.html>) applied to conduct the extraction and subsequent characterization of SHA (Aguirre et al., 2009; Mora et al., 2010; Olaetxea et al., 2015). A summary of data related to the physico-chemical characterization of SHA is presented in supplementary information (Table S1; Figures S1 and S2).

### 2.2 | Plant growth conditions and experimental design

Seeds of cucumber (*Cucumis sativus* L. cv "Ashley") were germinated in water with 1 mM of CaSO<sub>4</sub>, in darkness, on perlite and moistened filter paper in a seed germination chamber. One week after germination, plants were transferred to 8 L receptacles in hydroponic solution. The nutrient solution used contained the following compounds: 0.63 mM K<sub>2</sub>SO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM CaSO<sub>4</sub>, 0.30 mM MgSO<sub>4</sub>, 0.25 mM KNO<sub>3</sub>, 0.05 mM KCl, 0.87 mM Mg(NO<sub>3</sub>)<sub>2</sub>, 40 μM H<sub>3</sub>BO<sub>3</sub>, 4 μM MnSO<sub>4</sub>, 2 μM CuSO<sub>4</sub>, 4 μM ZnSO<sub>4</sub>, and 1.4 μM Na<sub>2</sub>MoO<sub>4</sub>. The nutrient solution contained 40 μM of iron as Fe-EDDHA chelate (80% ortho-ortho isomer). No precipitation of Fe inorganic species was observed throughout the experiment. The pH of the nutrient solutions was held at 6.0 and did not change significantly during the experiment. All experiments were performed in a growth chamber at 21/25 °C, 70%–75% relative humidity, and with 15/9 hr day/night photoperiod (irradiance: 250 μmol/m<sup>2</sup> s<sup>-1</sup>).

SHA was dissolved in 0.1 M NaOH before treatments. The concentration of SHA employed in the experiments (100 mg/L of organic carbon) was selected from the results obtained in previous studies (Mora et al., 2010; Olaetxea et al., 2015).

At these conditions, the following experiments were undertaken:

1. Validation of the experimental model: Effects of SHA on root PM H<sup>+</sup>-ATPase activity, Lp, and the upstream hormonal signals (IAA and ABA) were investigated. After ten days of growth in hydroponics, SHA treatment (100 mg/L of organic carbon) was applied to plant roots. The following measurements were undertaken over time: shoot and root dry weight; Lp; H<sup>+</sup>-ATPase activity in roots and confirmation of H<sup>+</sup>-ATPase involvement by measuring the cell type-specific H<sup>+</sup>-ATPase isoforms genetic

**TABLE 1** Shoot and root dry weights (DW) for plants with different treatments: (A) PM H<sup>+</sup>-ATPase inhibitor (DCC) and the combined treatment of DCC + SHA compared to control and SHA treatments. (B) ABA biosynthesis inhibitor treatment (Fld) and the combination of Fld + SHA compared again to control and SHA treatments. (C) Cytokinin action blocker, PI-55, and the combined treatment of PI-55 + SHA compared to control and SHA treatments, which was only explored in shoot

Treatment	Plant part	DW (mg)	
		72 hr	DW (%)
<b>A</b>			
Control	Shoot	377 ± 117 b	100%
SHA		508 ± 120 a	135%
DCC		342 ± 30 b	90%
DCC + SHA		335 ± 80 b	89%
Control	Root	116 ± 49 b	100%
SHA		177 ± 43 a	153%
DCC		90 ± 34 b	78%
DCC + SHA		79 ± 25 b	68%
<b>B</b>			
Control	Shoot	310 ± 45 b	100%
SHA		412 ± 99 a	133%
Fld		309 ± 69 b	100%
Fld + SHA		303 ± 100 b	98%
Control	Root	52 ± 7 b	100%
SHA		79 ± 36 a	152%
Fld		69 ± 28 b	133%
Fld + SHA		66 ± 30 b	127%
<b>C</b>			
Control	Shoot	319 ± 75 b	100%
SHA		461 ± 108 a	145%
PI-55		350 ± 55 ab	109%
PI-55 + SHA		418 ± 49 ab	131%

Note:  $p < .05$  (Fisher LSD). Harvests were conducted after 72 hr of treatment. Plant growth values are expressed as the mean value ( $n = 5$ ) ± standard deviation (SD). Treatments not sharing common letters are significantly different from one another ( $p < .05$ ) based on LSD Fisher *post hoc* test.

expression; and concentration of ABA and IAA in roots and cytokinins in the shoot (results are presented in Tables 1 and 2, Figure 1).

2. Evaluation of the relevance of the SHA-mediated activation of root PM H<sup>+</sup>-ATPase on the root and shoot growth-promoting effects of SHA by the application of the PM H<sup>+</sup>-ATPase inhibitor N, N' dicyclohexyl-carbodiimide (DCC). In this experiment, four plant treatments were applied after ten days of growth in hydroponics: control plants which continued to grow in nutrient solution for the duration of the experiment; SHA-treated plants (100 mg/L of organic carbon added to the nutrient solution); DCC-treated plants (5 μM of DCC inhibition treatment for a period of 30 min); and DCC + SHA combined treatment (initial treatment of 5 μM of DCC

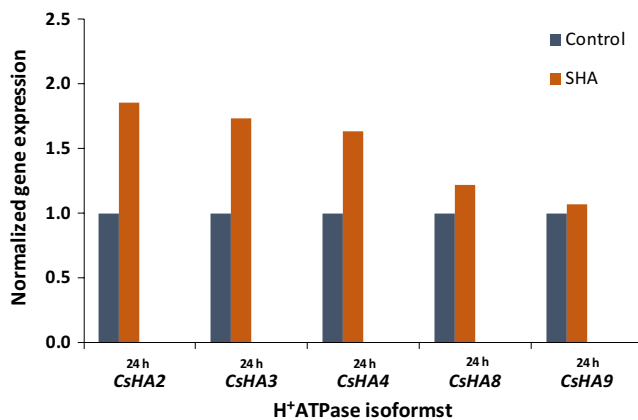
**TABLE 2** Evaluation of SHA-mediated growth-promoting action on plant hormone biosynthesis (root IAA, root ABA, and shoot cytokinins (CKs)), H<sup>+</sup>-ATPase activity, and on L<sub>p</sub>, under different treatments. (A) Control plants and SHA-treated plants; (B) PM H<sup>+</sup>-ATPase inhibitor (DCC) and the combination of DCC + SHA; (C) ABA biosynthesis inhibitor treatment (Fld) and the combination of Fld + SHA; and (D) Cytokinin action blocker, PI-55, and the combined treatment of PI-55 + SHA

Treatment	H <sup>+</sup> ATPase activity (mg pro <sup>-1</sup> min <sup>-1</sup> )		L <sub>p</sub> (g H <sub>2</sub> O g <sup>-1</sup> root DW Mpa <sup>-1</sup> hr <sup>-1</sup> )		Root IAA (pmol/g FW)		Root ABA (pmol/g FW)		Shoot CKs (pmol/g FW)		
	72 hr	72 hr	72 hr	72 hr	72 hr	72 hr	72 hr	72 hr	48 hr	48 hr	
A Control	0.44 ± 0.01 b	18.2 ± 2.7 b	40.0 ± 6.4 b	2.2 ± 0.1 b	2.0 ± 0.1 b	SHA	0.53 ± 0.01 a	29.3 ± 1.8 a	72.6 ± 5.3 a	3.0 ± 0.2 a	3.2 ± 0.2 a
B DCC	0.24 ± 0.08 a	4.7 ± 1.8 b	48.7 ± 3.3 b	3.0 ± 0.1 b*	3.5 ± 0.1 a	DCC + SHA	0.19 ± 0.07 a	16.4 ± 4.2 a	68.6 ± 5.0 a	3.9 ± 0.01 a*	3.0 ± 0.2 a
C Fld	0.34 ± 0.01 b	11.3 ± 0.5 a	27.1 ± 0.6 b	1.7 ± 0.1 a	1.1 ± 0.1 b <sup>#</sup>	Fld + SHA	0.45 ± 0.05 a	4.6 ± 0.3 b	42.1 ± 2.5 a	1.7 ± 0.3 a	1.7 ± 0.3 a <sup>#</sup>
D PI-55	ND	ND	31.0 ± 2.5 b	3.2 ± 0.1 b	2.3 ± 0.1 b	PI-55 + SHA			44.4 ± 1.5 a	3.7 ± 0.1 a	3.0 ± 0.2 a

Note:  $p < 0.05$  (Fisher). ND Not determined. All parameters were compared in the same plant tissue. The values are expressed as the mean value ( $n = 5$ ) ± standard error (SE). Significant differences are indicated between two treatments (control and SHA treatment) for experiments A, B, C, and D. Treatments not sharing common letters are significantly different from one another ( $p < .05$ ), according to LSD Fisher *post hoc* test.

\*Significant differences after 24 hr of treatment.

<sup>#</sup>Significant differences after 72 hr of treatment.



**FIGURE 1** SHA growth-promoting action involves increase in the gene expression of different cell type-specific H<sup>+</sup>-ATPase isoforms. Root cell type-specific H<sup>+</sup>-ATPase isoforms gene expressions (CshA2, CshA3, CshA4, CshA8, and CshA9) were measured. All data were standardized using *glutamyl-tRNA reductase* (HEMA1) and *alpha-tubulin* (*tua* gene) genes in each sample. No significant differences were found between treatments ( $p < .05$ )

for a period of 30 min and addition of 100 mg/L of organic carbon from SHA to a new nutrient solution after removing the DCC solution). The following parameters were measured over time as specified below: shoot and root dry weight, L<sub>p</sub>, H<sup>+</sup>-ATPase activity, ABA and IAA concentrations in roots, and cytokinin concentrations in the shoot (results are presented in Tables 1A and 2).

- Evaluation of the relevance of the SHA-mediated ABA-L<sub>p</sub> activation to the root and shoot growth-promoting effects of SHA by the application of fluridone, an ABA biosynthesis inhibitor. After growing plants in the conditions described above during 10 days, the following treatments were applied: control with only nutrient solution,

SHA treatment (100 mg/L of organic carbon), fluridone (Fld) treatment (10 μM of Fld), and treatment combining Fld (10 μM) and SHA (100 mg/L of organic carbon) (Fld + SHA). The following parameters were measured: shoot and root dry weight, L<sub>p</sub>, H<sup>+</sup>-ATPase activity, ABA and IAA concentrations in roots, and cytokinin concentration in the shoot (results are presented in Tables 1B and 2.)

- Evaluation of the relevance of the SHA-mediated increase in cytokinin concentration in leaves to the shoot growth-promoting action of SHA by applying a cytokinin action blocker, PI-55 (Spichal et al., 2009). The following treatments were studied after the initial 10 days of growth period: control plants (only nutrient solution), SHA-treated plants (100 mg/L of organic carbon), plants treated with cytokinin action blocker (PI-55 10 μM), and the final treatment combining PI-55 and SHA. The following parameters were measured at 24, 48, and 72 hr: shoot and root dry weight, root IAA and ABA concentrations, as well as the cytokinin concentration in leaves (results are presented in Tables 1C and 2.)

### 2.3 | Analysis of plasma membrane H<sup>+</sup>-ATPase activity in roots

Plasma membrane (PM) vesicles were isolated from apical roots (3–5 cm) using a sucrose-gradient technique as previously described by Mora et al. (2010). Briefly, the root segments corresponding to the different treatments were cut separately and ground with a pestle in an ice-cold homogenization medium containing: 250 mM sucrose, 10% (v/v) glycerol, 10 mM glycerol-1 phosphate, 2 mM MgSO<sub>4</sub>, 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT (dithiothreitol), 5.7% (w/v) choline-iodine, 1 mM PMFS, 20 μg/ml chymostatin, and 25 mM BTP (1,3-bis [TRIS (hydroxyl methyl) methyl aminopropane]

buffered to pH 6.7 with MES. Approximately, 2.5 ml/g fresh weight of root tissues was used. The homogenates were filtered through four layers of cheese cloth and subjected to 3 min of centrifugation at 13,000 g and 4°C (Beckman Coulter Microfuge 22R Centrifuge). The recovered supernatant (the liquid lying above the solid residue after centrifugation) was then centrifuged again at 13,000 g and 4°C for a further 25 min in order to obtain a microsomal membrane pellet (mass of substance). The pellets were then recovered, gently resuspended in 400 ml of homogenization medium, and loaded onto discontinuous density gradients made by layering 700 ml of 25% (w/w) sucrose over 300 ml of 38% (v/v) sucrose cushion in 1.5 ml tubes. The linear sucrose gradient from 20% to 55% (1.09–1.26 g/cm<sup>3</sup>) is generally used to separate membranes with different densities: tonoplast (1.10–1.12 g/cm<sup>3</sup>), Golgi membranes (1.12–1.15 g/cm<sup>3</sup>), rough endoplasmic reticulum (1.15–1.17 g/cm<sup>3</sup>), thylakoids (1.16–1.18 g/cm<sup>3</sup>), plasma membrane, (1.14–1.17 g/cm<sup>3</sup>), and mitochondrial membranes (1.18–1.20 g/cm<sup>3</sup>) (Yang & Murphy, 2013). Both sucrose solutions were prepared in 5mM BTP MES, pH 7.4, and contained all the protectants present in the homogenization medium. The gradients were centrifuged for 1 hr at 13,000 g, and the vesicles banding at the 25%–38% interface (1.14–1.17 g/cm<sup>3</sup>) were collected, diluted, and prepared for enzyme activity measurements.

The PM H<sup>+</sup>-ATPase activity was measured according to Mora et al. (2010). Assays were performed at 38°C in a 0.6 ml reaction vol. containing 50 mM MES-BTP, pH6.5, 5 mM MgSO<sub>4</sub>, 100 mM KNO<sub>3</sub>, 600 mM Na<sub>2</sub>MoO<sub>4</sub>, 1.5mM NaN<sub>3</sub>, 5 mM ATP-BTP, pH6.5, 0.01%(w/v) Brij58 (polyoxyethylene 20 cetyl ether), plus or in the absence of 100 mM V<sub>2</sub>O<sub>5</sub>. The reaction started with the addition of membrane vesicles (0.5 mg of total protein), and after 30 min, the reaction was blocked and the color developed. Inorganic phosphate concentration was determined spectrophotometrically at 705 nm.

## 2.4 | Measurement of root hydraulic conductivity (L<sub>pr</sub>) in the absence of hydrostatic pressure gradients (free exudation method)

When referring to the quantification of water uptake in plants, the major parameter reflecting root water uptake ability is root hydraulic conductivity (L<sub>p</sub>) (Martínez-Ballesta, Alcaraz-López, Mota-Cadenas, Muries, & Carvajal, 2011). L<sub>p</sub> concept was expressed as: g of exuded sap/g root DW \* time<sup>-1</sup> \* ΔMPa (osmotic pressure differences between nutrient solution and exuded sap). Thus, this methodology was applied in order to make the measurements. Stems were first cut from just below the first leaf. Then, the top part of the stem was introduced into a silicone tube and sealed with a self-sealing film to avoid any loss of sap. Xylem sap was finally collected with a glass Pasteur pipette. Collections were done continuously during the first 90 min of exudation after 72 hr of treatment and kept in a previously weighed 1.5 ml tube (in order to measure the mass of the sap and the osmotic pressure difference against the nutrient solution).

Measurement of the osmotic pressure of exuded sap:

The osmolality of the exuded sap and the nutrient solution was measured using a freezing point depression osmometer (Osmomat

010 Gonotec). Osmolality (mOsmol) was converted to osmotic pressure (MPa) by applying some equations (Bigot and Boucaud, 1998). The final root hydraulic conductivity (L<sub>pr</sub>) parameter was defined as mentioned above: g of exuded sap/g root DW \* time<sup>-1</sup> \* ΔMPa.

## 2.5 | Analysis of ABA and IAA concentrations in plants tissues

Plant endogenous IAA and ABA concentrations were analyzed using high-performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). Indole-3-acetic acid (IAA) and cis,trans-abscisic acid (ABA) reactants as well as the deuterium-labeled internal standards <sup>2</sup>H<sub>5</sub>-indole-3-acetic acid (D-IAA) and <sup>2</sup>H<sub>6</sub>(+)-cis,trans-abscisic acid (D-ABA) were purchased from OIChem Ltd.

The extraction and purification of the hormones of plant samples were carried out using the following method: 0.25 g of frozen plant tissue (previously ground to a powder in a mortar with liquid N<sub>2</sub>) was homogenized with 2.5 ml of precooled (-20°C) methanol:water:HCOOH (90:9:1, v/v/v, with 2.5 mM Na-diethyldithiocarbamate) and 25 μl of a stock solution of 1,000 ng/ml of deuterium-labeled internal standards in methanol. Extraction was performed by shaking the samples for 60 min at 2000 rpm at room temperature in a Multi Reax shaker. After extraction, solids were separated by centrifugation at 20,000 RCF for 10 min using a Sigma 4-16K Centrifuge, followed again by re-extraction with an additional 1.25 ml of extraction mixture by shaking for 20 min and centrifugation. About 2 ml of the pooled supernatants was separated and evaporated at 40°C using a RapidVap Evaporator. The residue was redissolved in 500 μl of methanol/0.133% acetic acid (40:60, v/v) and centrifuged at 20,000 RCF for 10 min before the injection into the HPLC-ESI-HRMS system.

Hormones were quantified using a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific), equipped with an HESI(II) source, a quadrupole mass filter, a C-Trap, a HCD collision cell, and an Orbitrap mass analyzer. A reverse-phase column (Synergi 4 mm Hydro-RP 80A, 150 × 2 mm; Phenomenex) was used. A linear gradient of methanol (A), water (B), and 2% acetic acid in water (C) was used: 38% A for 3 min, 38% to 96% A in 12 min, 96% A for 2 min, and 96% to 38% A in 1 min, followed by a stabilization time of 4 min. The percentage of C remained constant at 4%. The flow rate was 0.30 ml/min, the injection volume was 40 μl, and column and sample temperatures were 35 and 15°C, respectively. Ionization source working parameters were optimized and are reported in Table S2.

The detection and quantification of IAA and ABA were carried out using a Full-MS experiment with MS/MS confirmation in the negative ion mode, employing multilevel calibration curves with the internal standards. MS<sup>1</sup> extracted from the Full-MS spectrum is used for quantitative analysis, and MS<sup>2</sup> is used for the confirmation of targets identity. For Full-MS, a m/z scan ranging from 62 to 550 was chosen, and the resolution was set at 70,000 FWHM, the automatic gain control (AGC) target at 1·e<sup>6</sup>, and the maximum injection time (IT) at 250 ms. A mass tolerance of 5 ppm was accepted. The MS/MS

confirmation parameters are: resolution of 17,500 FWHM, isolation window of 3.0 m/z, AGC target of  $2 \cdot e^5$ , maximum IT of 60 ms, loop count of 1, and minimum AGC target of  $3 \cdot e^3$ . Instrument control and data processing were carried out by TraceFinder 3.3 EFS software. Accurate masses (m/z) for the phytohormones and their internal standards as well as for the principal fragments of these molecules are reported in Table S3.

## 2.6 | Analysis of cytokinin concentrations in leaf tissues

The following cytokinins were studied: trans- and cis-zeatin (tZ and cZ), dihydrozeatin (DHZ), trans- and cis-zeatin riboside (tZR and cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), benzyladenosine (BAR), meta-topolin (mT), meta-topolin riboside (mTR), ortho-topolin (oT), and ortho-topolin riboside (oTR). The deuterium-labeled internal standards  $^2H_5$ -trans-zeatin (D-tZ),  $^2H_5$ -trans-zeatin riboside (D-tZR),  $^2H_6$ -isopentenyladenine (D-iP),  $^2H_6$ -isopentenyladenosine (D-iPR),  $^2H_7$ -benzyladenosine (D-BAR), and  $^{13}C_5$ -ortho-topolin ( $^{13}C$ -oT) were used. All the standards were purchased from OIChemim Ltd.

Endogenous cytokinins in plants were analyzed using high-performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). Their extraction and purification were made using the method described by Dobrev and Kaminek (2002) with some variations: 0.25 g of frozen plant material (previously ground in a mortar to a powder with liquid nitrogen) was homogenized with 4 ml of precooled ( $-20^\circ C$ ) methanol-water-formic acid (15:4:1, v/v/v) solution. Deuterium-labeled internal standards were added to the extraction medium (25  $\mu$ l of a stock solution of 100 ng/ml of each standard in methanol). After overnight extraction at  $-20^\circ C$ , solids were separated by centrifugation at 20,000 RCF for 10 min at  $4^\circ C$  using a Sigma 4-16K Centrifuge (Sigma Laborzentrifugen GmbH), and re-extracted with an additional 2 ml of extraction mixture by shaking for 20 min in a Multi Reax shaker (Heidolph Instruments) and centrifugation. Supernatants were passed through a Sep-Pak  $C_{18}$  cartridge (ref. WAT054945, Waters Co.) preconditioned with 2 ml of methanol and 2 ml of extraction medium. The eluted material was evaporated to near dryness using a RapidVap Evaporator (Labconco Co.), and the residue was redissolved in 2 ml of 1M formic acid and applied to an Oasis MCX column (ref. 186000254, Waters Co.) preconditioned with 2 ml of methanol and 2 ml of 1M formic acid. The column was washed successively with 2 ml of 1M formic acid, 2 ml of methanol, and 2 ml of 0.35M  $NH_4OH$ , and the cytokinin bases and ribosides were eluted with 2 ml of 0.35M  $NH_4OH$  in 60% methanol (v/v). This eluted material was evaporated to dryness in the RapidVap Evaporator and redissolved with 250  $\mu$ l of methanol +250  $\mu$ l of 0.04% formic acid and centrifuged at 20,000 RCF for 10 min before the injection in the HPLC-ESI-HRMS system.

Cytokinins were quantified using a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific), equipped with an HESI(II) source, a quadrupole mass filter, a C-Trap, a HCD collision cell, and an

Orbitrap mass analyzer. A reverse-phase column (Tracer Excel 120 ODSA 3  $\mu$ m,  $200 \times 4.6$  mm, Teknokroma) was used. A linear gradient of methanol (A), water (B), and 0.4% formic acid in water (C) was used: time 0 min 45% A, 45% to 95% A in 14 min, 95% A for 0.5 min, and 95% to 45% A in 0.5 min, followed by a stabilization time of 6 min. The percentage of C remains constant at 5%. Flow rate was 0.40 ml/min, the injection volume was 20  $\mu$ l, and column and sample temperatures were 30 and  $15^\circ C$ , respectively. Ionization source working parameters were optimized and are reported in Table S4.

The detection and quantification of the cytokinins were carried out using a PRM experiment (Parallel Reaction Monitoring) in the positive ion mode, employing multilevel calibration curves with the internal standards. For each cytokinin, two fragment ions were analyzed. The fragment ion with the higher intensity (fragment 1) was used for quantification, and the other ion (fragment 2) was used for confirmation of target identity. In the case of internal standards, only the fragment ion of the higher intensity was analyzed. The resolution was set at 35,000 FWHM, the automatic gain control (AGC) target at  $2 \cdot e^5$ , and the maximum injection time (IT) at 125 ms. The collision energy (CE) depends on the molecule. A mass tolerance of 5 ppm was accepted. Instrument control and data processing were carried out by TraceFinder 3.3 EFS software.

Accurate masses (m/z) of the phytohormones and their internal standards as well as the principal fragments of these molecules were quantified. Collision energy (CE) for each of the molecules is reported in Table S5.

## 2.7 | Reverse transcription-qPCR analysis of RNA transcript

The roots of the plants were collected and disrupted with liquid nitrogen prior to RNA extraction. Total RNA was extracted from crushed root weighing between 50 and 90 mg using a mixture of 350  $\mu$ l of guanidinium-thiocyanate lysis buffer and 3.5  $\mu$ l of  $\beta$ -mercaptoethanol from the NucleoSpin RNA Plant Kit (Macherey-Nagel). Treatment of RNA with DNase was performed according to the manufacturer's recommendations. After washing the extracted RNA with dry silica membranes provided by the kit, RNA purity and concentration were quantified by the fluorescence-based Experion RNA STdSens Analysis kit. First-strand cDNA synthesis was carried out in 20- $\mu$ l reactions containing 1  $\mu$ g of RNA with RNase H-MMLV (Human Moloney murine, leukemia virus) reverse transcriptase iScript and a mix of oligo(dT) and random hexamer primers from iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The reverse transcription was carried out for 5 min at  $25^\circ C$ , 30 min at  $42^\circ C$ , and finally for 5 min at  $85^\circ C$ . Reverse transcription-qPCR analysis was performed using iQ SYBR Green supermix containing hot-start iTaq DNA polymerase in an iCycler iQ (Bio-Rad Laboratories). Primer pairs used to amplify cucumber plasma membrane  $H^+$ -ATPase isoforms were taken from previously published work (Wdowikowska & Klobus, 2016) and synthesized by Condalab (Madrid, Spain). Standardization was carried

out based on the expression of the two *Cucumis sativus* reference genes: *glutamyl-tRNA reductase* (*HEMA1*) (Acc. No. MF033082.1) and *alpha-tubulin* ( $\alpha$ -*tua* gene) (Acc. No. AJ715498.1) (Migocka & Papierniak, 2011; Wan et al., 2010; Warzybok & Migocka, 2013).

The RT-qPCR program consists of an iTaq DNA polymerase activation at 95°C for 3 min, followed by 40 amplification cycles: denaturing step for 10 s at 95°C, an annealing step for 10 s (maintained at different temperatures between 54.5°C and 61°C depending gradient temperature for each pair of primers), and an elongation step for 20 s at 72°C during which the fluorescence data were collected. To confirm the PCR products, a melting curve analysis was performed by heating the samples from 72 to 95°C in 0.5°C increments with a dwell time at each temperature of 10 s during which the fluorescence data were collected. Data analysis of the relative abundance of the transcripts was done using CFX Manager Software Data Analysis (Bio-Rad Laboratories). Expression analyses were carried out in five independent root RNA samples and repeated three times for each RNA sample. The two reference genes selected for these experiments showed CV values between 0.25 and 0.50, which means that our reference genes were between homogeneous and heterogeneous sample panels.

## 2.8 | Statistical analysis

Significant differences ( $p \leq .05$ ) among treatments were calculated by using one-way analysis of variance (ANOVA) and the LSD Fisher *post hoc* tests. All statistical tests were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa USA).

## 3 | RESULTS

### 3.1 | Experimental model validation

In order to validate our experimental model, we first verified that SHA root application is associated with the different events obtained in previous studies (Mora, Bacaicoa, et al., 2014a; Mora et al., 2012; Olaetxea et al., 2015).

SHA-treated plants showed significant differences in shoot and root dry weight after 72 hr of the treatment (Table 1). Furthermore, the root PM H<sup>+</sup>-ATPase activity,  $Lp_r$ , and root IAA concentration significantly increased after 72 hr of treatment for SHA-treated plants compared to control plants (Table 2, A). Also, SHA-treated plants showed significant differences in root ABA concentration 72 hr after the onset of SHA treatment (Table 2, 2). These results confirm the suitability of our plant model to study the mechanisms involved in the plant growth-promoting effect of root-applied SHA.

In addition to the activity of root PM H<sup>+</sup>-ATPase, the expression of genes belonging to the root PM H<sup>+</sup>-ATPase genes family in cucumber (Wdowikowska & Klobus, 2016) was also measured. SHA-treated plants showed increases in the expression of CsHA2, CsHA3, CsHA4, CsHA8, and CsHA9 genes compared to control plants 24 hr after the onset of SHA treatment. However, these differences were not significant (Figure 1).

### 3.2 | Regulation of root PM H<sup>+</sup>-ATPase activity is crucial for the SHA-mediated increase in plant growth

In order to determine the relevance of the SHA-mediated increase to root PM H<sup>+</sup>-ATPase activity in the mechanism of action of SHA on plant growth, an H<sup>+</sup>-ATPase inhibitor, N, N' dicyclohexyl-carbodiimide (DCC), was employed in the experiments. The results showed that DCC application prevented the increase in both root and shoot growth caused by SHA, thus indicating that PM H<sup>+</sup>-ATPase stimulation is a crucial step for the plant growth-promoting action of SHA (Table 1, B). In contrast, the upstream SHA-mediated increase in IAA root concentration was observed in plants treated with both SHA and SHA + DCC (Table 2, B).

On the other hand, SHA caused an increase in  $Lp_r$  in the presence of DCC after 72 hr upon SHA treatment (Table 2, B). These results were accompanied by an upstream increase in root ABA concentration for both SHA and SHA + DCC treatments (Table 2, B).

The results indicate that the SHA effects mediated by the IAA-root PM H<sup>+</sup>-ATPase pathway are independent of the ABA- $Lp_r$  pathway.

### 3.3 | SHA-mediated increases in root ABA concentration and $Lp_r$ are essential for the SHA-mediated root growth-promoting action

The application to plant roots of an inhibitor of ABA biosynthesis (FId) inhibited the increase in shoot growth caused by SHA (Table 1, B). This experiment also showed that the SHA-mediated increase in root growth is also inhibited by FId (Table 1, B). On the other hand, FId application prevented the SHA-mediated increase in  $Lp_r$  (Table 2, C). All these results were in good agreement with root ABA biosynthesis that remained inhibited by FId + SHA (Table 2, C). However, FId did not affect the increase of both root IAA concentration and root PM H<sup>+</sup>-ATPase activity caused by SHA (Table 2, C).

### 3.4 | The SHA-mediated increase in shoot cytokinin concentration is needed for the shoot growth-promoting action of SHA, and is regulated through the IAA-PM H<sup>+</sup>-ATPase signaling pathway

In order to elucidate the role played by the SHA-mediated increase in shoot cytokinin concentration and in turn shoot growth, an inhibitor of the cytokinin signaling pathway (PI-55) was applied along with SHA. The results showed that the addition of an inhibitor of the cytokinin signaling pathway (PI-55) was able to prevent the SHA stimulating effect (Table 2, D), but it is important to note that this treatment alone had a stimulating effect on plant shoot growth. Nevertheless, cytokinin concentration was significantly increased in the shoot of plants treated with SHA and PI-55 compared to PI-55 treatment (Table 2, D). Regarding the possible role of root PM H<sup>+</sup>-ATPase activity and  $Lp_r$  in the SHA-mediated increase in shoot cytokinin concentration, DCC application inhibited the shoot cytokinin increase caused by SHA (Table 2, B). However,

Fld did not affect the SHA-mediated increase of shoot cytokinin concentration (Table 2, C). As expected, PI-55 did not affect the upstream increases in IAA and ABA root concentrations caused by SHA (Table 2, D).

## 4 | DISCUSSION

Many studies have demonstrated the presence of high correlation between soil fertility, crop productivity, and the content of transformed (humified) natural organic matter in the soil (Chen et al., 2004; Stevenson, 1994). The beneficial effects of humified organic matter, principally HS, on plant growth are driven by improvements in plant mineral nutrition and rhizosphere physicochemical features (Aguirre et al., 2009; Chen et al., 2004; Olaetxea et al., 2018). In some cases, direct effects of HS on plant metabolism and physiology may also be involved (Nardi, Pizzeghello, Muscolo, & Vianello, 2002; Olaetxea et al., 2016, 2018; Vaughan & Malcolm, 1985). While the mechanisms underlying the effects of HS on soil nutrient bioavailability and rhizospheric physicochemical features are relatively well-known (Chen et al., 2004; Garcia-Mina, 2006), those responsible for the direct effects of HS on plant development are unclear (Nardi et al., 2002; Olaetxea et al., 2018). Many studies have reported several molecular and biochemical events that occur as a result of HA root application, but there is a lack of experimental evidence showing the integration of these events into the whole mechanism of action of HA on plant growth (Olaetxea et al., 2018 and references therein). One of these events, caused by root-applied HA in plants, is the increase in root PM  $H^+$ -ATPase activity (Olaetxea et al., 2018 and references therein).

### 4.1 | Root PM $H^+$ -ATPase activity and root ABA play a crucial role in the root growth-promoting action of SHA

With regard to the HA effects on root morphology and growth, a number of studies have reported that HA are able to mimic the effects caused by natural auxins, such as IAA, on root development (Nardi et al., 2002; Olaetxea et al., 2018). In fact, the use of inhibitors of auxin action inhibited some of the effects caused by HA on lateral root development, as well as the HA-mediated expression of genes also regulated by auxin-dependent signaling pathways (Trevisan, Pizzeghello, et al., 2010b). In agreement with these results, HA root application produced significant increases in the concentration of root IAA, which were also linked to concomitant increases in ethylene and NO in the root (Mora et al., 2012). Although some of the effects caused by HA on root architecture and lateral root development are explained by the effects of HA through auxin- and NO- dependent pathways (Zandonadi et al., 2010), the HA-mediated increase in the whole root growth measured by dry matter production was independent of IAA, ethylene, or NO (Mora et al., 2012). Some authors have proposed that

the whole effect of HA on root growth is mediated by the capacity of HA to promote root PM-  $H^+$ -ATPase activity under the acid growth theory (Ramos et al., 2015). However, to our knowledge, there are no mechanistic studies involving plant mutants with no, or very low,  $H^+$ -ATPase activity or specific inhibitors of  $H^+$ -ATPase activity, allowing the demonstration of whether the HS-mediated increase in root PM-  $H^+$ -ATPase activity is essential for the HS-promoting action of root growth. We have studied here the effect of DCC, an inhibitor of  $H^+$ -ATPase activity, on the SHA capacity to enhance root growth. Our results clearly show that the presence of DCC prevents the SHA-mediated increase in both root PM  $H^+$ -ATPase activity and root growth (Table 1, B and Table 2, B). This fact indicates that the capacity of SHA to increase root PM-  $H^+$ -ATPase activity is directly involved in its promoting effect on root growth.

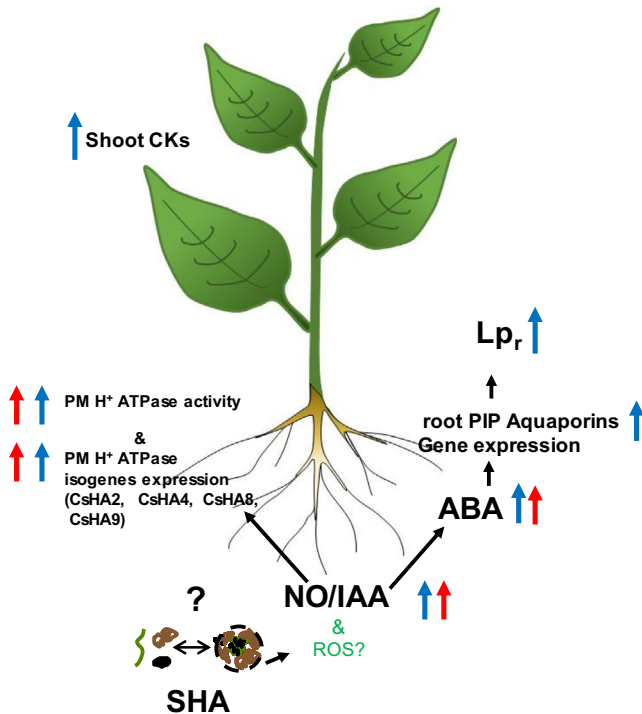
On the other hand, Olaetxea et al. (2015) reported that SHA was able to increase  $Lp_r$  through ABA-dependent pathways, with this effect being essential for the enhancement of shoot growth. In this sense, recent studies have shown that root ABA affects root growth and architecture by interacting with some nitrate transporters (Harris & Ondzighi-Assoume, 2017). Several studies have shown that HA with diverse origins increased the expression of nitrate transporters in different plant species (Olaetxea et al., 2018 and references therein). It is therefore possible that the capacity of SHA to enhance root growth also involves root ABA-dependent signaling pathways. Our results presented here confirm this hypothesis. The application of Fld, an inhibitor of ABA synthesis but not of ABA perception, significantly decreased the SHA-mediated enhancement of root growth (Table 1, B). This effect was associated with a significant decrease in  $Lp_r$  (Table 2, C). This fact indicates that the SHA-mediated increase in root ABA concentration also plays a relevant role in the root growth-promoting action of SHA. These results are in line with Harris and Ondzighi-Assoume (2017) who showed the relevant role of root ABA in the modulation of root growth. Our results indicate that whereas some aspects of the SHA-mediated effects on root development (lateral and adventitious root proliferation) and architecture (length of principal root) are expressed through IAA, NO, and ethylene signaling pathways (Mora et al., 2012), other aspects concerning the whole root growth involve ABA signaling pathways.

The results presented here show that both root PM  $H^+$ -ATPase activity and root ABA play a crucial role in the growth-promoting action of SHA in cucumber, the plant species we used in this experiment (Figure 2).

### 4.2 | Besides ABA, ethylene, IAA, and NO, other still unknown signals are involved in the SHA effect on root development

ABA is probably not the only signal involved in the SHA-mediated effect on root growth. This conclusion is suggested by previous studies showing that both IAA and ethylene are involved in the increase of root ABA concentration caused by SHA in cucumber (Mora, Bacaicoa, et al., 2014a). However, the inhibition of IAA or ethylene





**FIGURE 2** Conclusion icon. Main pathways involved in the mechanism of action of sedimentary humic acid on plant growth-promoting effect. Blue arrows indicate SHA-mediated mechanism of action for shoot growth promotion, while red arrows describe the SHA-mediated root-promoting action, in our model plant (cucumber)

action did not prevent the SHA-mediated enhancement in root growth (Mora et al., 2012). This same reasoning might be applied to the regulation of root PM H<sup>+</sup>-ATPase activity, since IAA and NO are positive regulators of the action of HA on root PM H<sup>+</sup>-ATPase activity (Zandonadi et al., 2010), and the inhibition of the action of the two phytohormones did not prevent the SHA-mediated increase in cucumber root growth (Mora et al., 2012). These results might agree with each other if the SHA-mediated stimulation of root PM H<sup>+</sup>-ATPase activity and root ABA concentration requires the simultaneous action of several of the phytohormones affected by SHA (IAA, ethylene, and NO). In fact, the experiments described in Mora et al. (2012), Mora, Bacaicoa, et al. (2014a) did not involve inhibitors of the three phytohormones used simultaneously. In this line, taken together, these results may be indicative of a relevant role of the ratio between specific phytohormones (e.g., the IAA: active cytokinins ratio) rather than a singular or independent action of one of them.

A complementary role of other signals in the regulation of this process cannot be ruled out either (Schmidt, Santi, Pinton, & Varanini, 2007).

Among other possible signaling pathways, reactive oxygen species (ROS) might play a potential role. Several studies have reported that HA with diverse origin were able to modulate ROS concentration in roots (Berbara & García, 2014; García et al., 2012, 2016). García et al. (2016) demonstrated in rice that the application

of HA extracted from a vermicompost was able to cause a moderate increase in both hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sub>2</sub><sup>•-</sup>) levels in different parts of the root. This increase was accompanied by a coordinated increase in the activity of the main enzymes involved in the regulation of ROS homeostasis. All these effects were associated with a promotion of lateral root development. Although the relationships between ROS and PM H<sup>+</sup>-ATPase activity in plants is not very clear, recent studies have shown that blue light, a PM H<sup>+</sup>-ATPase activator, increased ROS generation in Arabidopsis, which might be the cause of H<sup>+</sup>-ATPase activation (El-Esawi et al., 2017). This result may support the hypothesis that ROS might be partially responsible for the effects on the rest of the plant that are related to PM H<sup>+</sup>-ATPase activity. In any case, new and specific studies are needed to validate this hypothesis, as well as to explore the role of other potential signals in the SHA-mediated activation of root PM H<sup>+</sup>-ATPase activity and plant growth.

#### 4.3 | The SHA-mediated stimulation of root PM H<sup>+</sup>-ATPase activity is needed for the SHA-promoting action on shoot growth

With regard to HS effects on shoot growth, Mora, Olaetxea, et al. (2014b) showed that SHA enhancement of shoot development in cucumber is mediated by an initial effect resulting in an increase in the IAA and NO levels in the roots. In fact, the use of inhibitors of IAA function or NO scavengers in that study prevented the SHA-mediated increase in shoot growth (Mora, Bacaicoa, et al., 2014a). A subsequent study also showed that both root ABA and Lp<sub>r</sub> are also involved in the regulation of the increase in cucumber shoot growth mediated by SHA through the activation of specific root PM aquaporins (Olaetxea et al., 2015). The results presented here show that the SHA-mediated activation of the root PM H<sup>+</sup>-ATPase activity also plays a role in the shoot growth-promoting action of SHA in cucumber, since the application of DCC—an inhibitor of root PM H<sup>+</sup>-ATPase activity—prevented the increase in shoot growth caused by SHA (Table 1, B). This result is in line with a previous study proposing that the enhancement in shoot growth caused by SHA in cucumber was related to the increase in the concentration of active cytokinins in the shoot associated with both root PM H<sup>+</sup> ATPase activity and nitrate root uptake (Mora et al., 2010). Our results support this hypothesis, showing that root PM H<sup>+</sup> ATPase activity plays a crucial role in this effect of SHA on shoot development. This hypothesis is also supported in our study by the results obtained in experiments involving a blocker of cytokinin perception, PI-55. The presence of PI-55 reduced the SHA-mediated increase in shoot growth (Table 1, D), although this compound had a plant growth-stimulating effect. This result is consistent with a relevant role of cytokinin in the action of SHA, and HA in general, on shoot growth and development. Furthermore, the presence of DCC prevented the SHA-mediated increase in shoot cytokinin concentration (Table 2, B). These results show that the SHA-mediated increase in root PM H<sup>+</sup>-ATPase



activity plays a crucial role in the mechanism underlying the increase in shoot cytokinin concentration caused by SHA, in good agreement with the hypothesis proposed by Mora et al. (2010).

The results indicate two chains of events triggered by the interaction of SHA with plant roots that are needed for the enhancement of shoot growth caused by SHA in cucumber: an increase in root PM H<sup>+</sup>-ATPase activity, which also mediates an increase in cytokinin concentration and action in the shoot, and an increase in root ABA concentration.

#### 4.4 | The increase in root PM H<sup>+</sup>-ATPase activity and the ABA-mediated increase in Lp<sub>r</sub> both caused by SHA, are independent of each other

A number of studies have shown the functional relationships between IAA, ethylene, ABA, and root PM H<sup>+</sup>-ATPase activity (Hager, 2003; Wdowikowska & Klobus, 2016). It is, therefore, possible that the SHA-mediated increase in both root PM H<sup>+</sup>-ATPase activity and root ABA concentration are functionally linked to each other. In order to investigate this possibility, we have studied the effect of the inhibition of the SHA-mediated increase in the root PM H<sup>+</sup>-ATPase activity on the effects of SHA on root ABA concentration and Lp<sub>r</sub>. The results showed that DCC did not inhibit the increase in both root ABA concentration and Lp<sub>r</sub> caused by SHA (Table 2, B). Likewise, experiments including Fld showed that the inhibition of root ABA biosynthesis did not affect the SHA-mediated increase in root IAA concentration, root PM H<sup>+</sup>-ATPase activity, and the concomitant increase in shoot cytokinin concentration (Table 2, C). These results show that the SHA-mediated increase in both root H<sup>+</sup>-ATPase activity and root ABA concentration are independent of each other, and that both are together necessary for the enhancement of plant growth caused by SHA in cucumber.

## 5 | CONCLUSION

The results presented here clearly show that both the root PM H<sup>+</sup> ATPase activity and root ABA are two major players in the mechanism underlying the prompt action of SHA in enhancing shoot and root growth in cucumber. These effects involve the upstream activation of IAA-, NO-, ethylene-, and ABA-dependent signaling pathways. In the case of the effects on the shoot, SHA action also involves a root PM H<sup>+</sup> ATPase-dependent increase in shoot cytokinin concentration and activity (Figure 2). In the case of the effects on the roots, other signaling pathways besides those involving IAA, NO, ethylene, and ABA are probably also involved. In this sense, ROS would be a suitable candidate to participate in this role, but further studies are necessary to confirm this hypothesis.

We do not have information about the relevance of the mechanisms associated with the short-term action of SHA on plant growth in the whole effect of this compound on plant development during the complete plant cycle. New and specific research must be carried out in order to better know the complex nutritional and metabolic

network that is involved in the whole beneficial action of HS on plant growth.

## ACKNOWLEDGMENTS

This study has been supported by Roullier Group, the Government of Navarra, and the University of Navarra.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest associated with the work described in this manuscript.

## AUTHOR CONTRIBUTIONS

M.O., R.B., and J.G-M. conceived the original screening and research plan; M.O. performed most of the experiments; A.Z., M.F., M.G., E.B., R.B., and V.M. provided technical assistance to M.O.; L.S. synthesized and provided reagents. M.O. and J.G-M. designed the experiments and analyzed the data. J.G. and M.O. conceived the project and wrote the article. M.O. and J.G-M. agree to serve as the authors responsible for contact and ensure communication.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Olaetxea M, Mora V, Bacacoa E, et al. Root ABA and H<sup>+</sup>-ATPase are key players in the root and shoot growth-promoting action of humic acids. *Plant Direct*. 2019;3:1–12. <https://doi.org/10.1002/pld3.175>