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# Humic acids and *Herbaspirillum seropedicae* change the extracellular H<sup>+</sup> flux and gene expression in maize roots seedlings

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## Abstract

**Background:** The use of plant-based biostimulants to increase crop yield and enhance protection has grown in popularity. We previously manufactured an inoculant combining humic acids with plant growth-promoting bacteria and observed a significant effect on crop yields. However, electrophysiology studies elucidating the mechanisms responsible for enhanced nutrient and water uptake are scarce and are generally restricted to in vitro models.

**Materials and methods:** In this study, a suspension of humic acids isolated from vermicompost along with *Herbaspirillum seropedicae* was applied to maize seedlings. We measured the root H<sup>+</sup> flux with an ion-selective vibrating probe system. Furthermore, the transcription of plasma membrane H<sup>+</sup> ATPase, aquaporin and high- or low-affinity nitrate transporters was measured.

**Results:** Inoculation activated the extracellular H<sup>+</sup> flux, thus changing the pH and membrane potential of maize root cells and altering the electrochemical potential generated by P-H<sup>+</sup>-ATPase at the biochemical and molecular level. The overexpression of aquaporins was also observed; however, nitrate transporters were repressed by the inoculants.

**Conclusion:** We demonstrate an increase in the H<sup>+</sup> efflux in maize root seedlings inoculated with *H. seropedicae* and concomitant changes in membrane voltage. The inoculation of *H. seropedicae* in the presence of humic acids decreased the H<sup>+</sup> flux and surface acidification without changes in the aquaporin transcription level. However, the H<sup>+</sup> flux in root seedlings in inoculated plants (with or without humic acids) was larger in respect to control plants. These results support the increased water and nitrogen efficiency in plants inoculated with humic acids and *H. seropedicae*.

**Keywords:** Humic substances, Plant growth-promoting bacteria, Diazotrophic endophytic bacteria, Physiological effects, Ecological intensification of crops

## Introduction

Humic substances (HS) comprise a major part of soil organic matter and influence soil processes and fluxes including soil fertility, water retention and the fate of xenobiotics [1]. HS can be used directly on plants in low concentrations to enhance plant growth, yield and nutrient uptake, thus constituting a category of plant

biostimulants [2]. The effects of HS on plant growth and crop yield have been studied previously [3]. HS enhanced the efficiency of nutrient use, thus aiding in the assimilation of both macro- and microelements and inducing primary and secondary plant metabolism [3]. It was reported that humic acids (HA) can stimulate plant growth through the release of bioactive molecules with hormonal-like activity [4], thus directly affecting enzymatic activities of several metabolic pathways [2, 5, 6]. The effect of HS on plant nutrition may be mediated by the synthesis and functionality of membrane proteins, especially proton pumps that increase the electrochemical proton gradient across the plasma membrane (P) [7,

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8]. The first report of proton pump stimulation by HS was provided by Maggioni et al. [9]. Due to its crucial role in ion uptake and root growth, this phenomenon has been well studied [10–17]. HA can increase the activity of plasma membrane (P)  $H^+$ -ATPase (P- $H^+$ -ATPase) and vacuolar  $H^+$ -ATPase, thus modulating the cellular electrical environment and proton pump activity that are used as biochemical markers of HA bioactivity [18].

Assuming that pH changes precede or reflect ongoing processes [19], pH could serve as a messenger where pH changes are needed for certain processes such as root growth [18]. The P- $H^+$ -ATPase could transcend its classical functions as an energy transducer system and assume a key role as a signal transducer [20]. The bioactive compounds in the HA supramolecular arrangement can be recognized by transmembrane receptor proteins [21, 22] or access the cytoplasm and thus modify the cytosolic pH ( $pH_{cit}$ ). Small variations in the  $pH_{cit}$  play a critical role in several cellular processes, including the induction of enzymatic activities and ion transport [23]. Indeed, the activation of the P-type  $H^+$ -ATPase increases the extracellular  $H^+$  flux, thus making the apoplast more acidic and providing an optimal medium for the activity of expansin proteins, which are fundamental for cell expansion [24, 25]. The activation of  $H^+$  flux is also crucial for energizing secondary transporters [8]. The enhancement of nutrient uptake, root growth promotion and absorption of water are among the main biostimulation effects of HS [26] and are responsible for the increase in crop yields in soils with low fertility [27, 28].

In highly weathered soils, low nitrogen is often cited as the main limiting factor in crop yield and results in the high cost of fertilizers. The use of plant growth-promoting bacteria (PGPB) is a sustainable alternative that enhances the efficiency of nutrient usage [28]. Biological nitrogen fixation and the solubilization of soil phosphorus are the main targets of commercial biostimulants that are manufactured using PGPB [29]. However, the success of PGPB in non-leguminous plants is substantially less than the results obtained in rhizobia–legume symbioses. In non-nodulating plant species, PGPB need to colonize epiphytic and endophytic tissues, and these processes are influenced by environmental stress and microbiological competition with native species.

The use of HA to introduce PGPB to crops has been documented [27, 30, 31]. Olivares et al. [32] reviewed the basic mechanisms and benefits of the combined application of HA and PGPB to several crop species. The suitability of HA as an adjuvant to bacterial foliar sprays was demonstrated for field-grown maize, where yields were 65% higher than untreated controls and 40% higher than

maize fields treated with HA or bacteria [27]. Moreover, a boost in the metabolic profile of sugarcane leaves induced by HA and PGPB [33] with activation of the glycolysis pathway and enhanced TCA activity resulted in the accumulation of  $\alpha$ -ketoglutaric acid, which is a central metabolite in glutamine and glutamic acid synthesis. The stimulation of TCA enzymes by HS was previously reported by Nardi et al. [34].

Regulation of the electrochemical environment at the cell/aqueous solution interface by HA and PGPB has not been well investigated. Numerous mechanisms have been shown to function in the PGPB–host plant interaction, including the alleviation of abiotic stress, siderophore production, the production of 1-aminocyclopropane-1-carboxylate deaminase (ACC), phytohormone production, antifungal activity, the induction of systemic resistance, and interference with pathogen toxin production [35]. The effect of PGPB alone or in combination with HA on proton pump activity has been minimally studied despite the influence on nitrate and water uptake. The aim of this work was to evaluate  $H^+$  flux in root cells treated with HA and PGPB and the effects on transcription of genes encoding nitrate transporters and aquaporins.

## Materials and methods

### HA obtainment and its chemical derivatives

The HA used in this study was isolated from the vermicompost of filter cake, which is a typical product of the sugar industry in the north of Rio de Janeiro State, Brazil. HA was obtained according to the classical method of extraction, isolation and purification described in the web page of the International Humic Substances Society (<http://www.ihss.gated.edu>). After freeze drying by lyophilization, the carbon content was analyzed by dry combustion (CHN analyzer Perkin Elmer series 2400, Norwalk, CT, USA). The chemical nature of HA was accessed by cross-polarization magic angle spinning (CP/MAS)  $^{13}C$  nuclear magnetic resonance ( $^{13}C$ -NMR). The spectrum was acquired from the solid sample with a Bruker Avance 500 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 spectra were 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).

### Obtainment of microbial inoculum

The bacterium used was *Herbaspirillum seropedicae* strain HRC54, which was originally isolated from sugarcane roots [36]. The pre-inoculum was prepared from a frozen bacterium stored at 15% glycerol. An aliquot of 50  $\mu\text{L}$  was grown in 5 mL tube containing liquid nutrient broth (NB) medium for 36 h at 30 °C under orbital shaker at 150 rpm and checked under phase contrast microscopy for contamination and later by colony morphology in JNFb solid medium. A 200 mL Erlenmeyer flask containing 50 mL of NB liquid medium was inoculated with 100  $\mu\text{L}$  of the pure culture pre-inoculum and was grown at the same condition reported above for 48 h. After that, the bacteria cells were harvested, centrifuged, washed, and the optical density adjusted to 1.8 at 460 nm (approximately  $10^9$  cells per mL).

### Plant growth and treatment with humic materials and PGPB

Maize seeds (*Zea mays* L. var. UENF 506-11) were disinfected through immersion in a solution of NaClO (1.0%) for 30 min. Then, the seeds were washed and placed in distilled water for a period of 6 h. After, seeds were germinated in paper, in the dark, at 28 °C. Four days after germination, maize seedlings with approximately 0.5–0.7 cm were transferred to pots containing a minimal medium solution (2 mM  $\text{CaCl}_2$ ) and the treatments were: 0 (control), 2 mM  $\text{C L}^{-1}$  of the HA, *Herbaspirillum seropedicae* strain HRC54 produced at a concentration of  $10^9$  viable cells per mL, according to Döbereiner et al. [37] (bacteria) or the combination of HA and bacteria (HA + B). The pH of the solution was adjusted to 5.8–6.0. After 7 days in hydroponic culture, the seedlings were harvested and the analyses were performed. The minimal medium (2 mM  $\text{CaCl}_2$ ) was used to avoid any influence of the nutrients, which could act synergistically with the HA, stimulating root growth and seedlings' metabolism [32].

### Dry mass and root length

After 7 days in hydroponics culture, roots were dried in forced air circulation oven at a temperature of 50 °C for 48 h and weighed on an analytical balance. Root length was measured with a digital caliper. Data are presented as an average of four repetitions.

### Extraction of total RNA

100–200 mg of fresh root tissues were homogenized with a mortar and pestle in the presence of liquid  $\text{N}_2$ . The homogenate was transferred to new RNase-free microcentrifuge tubes (1.5 mL) and the RNA was extracted using the mini-plant RNeasy Qiagen<sup>®</sup> kit (Germantown, USA).

### Reverse transcription (RT) followed by polymerase chain reaction (PCR)

1–2  $\mu\text{g}$  of total RNA was used for production of cDNAs. The synthesis was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). A PCR with a gradient temperature (59, 60 and 61 °C) was performed to confirm the specificity of the primers and the actual melting temperature. Electrophoresis in 2.0% agarose gel with TAE buffer was also performed for the confirmation of PCR products with the specific primers. Primers for the genes ubiquitin (UBI), aquaporin (PIP), low-affinity nitrate transporter (Nrt2.1), high-affinity nitrate transporter (Nrt1.1), PM- $\text{H}^+$ -ATPase (Mha1) were designed with Primer3 program and its characteristics were evaluated in Oligothench program and after a rigorous analysis, they were synthesized by IDT technology. Confirmation of primers specificity was obtained in a high-resolution gel, which gave single PCR products at the different temperatures tested and with the expected size. Specificity was also confirmed by melting curve performed in Step OneTMAppliedBiosystems<sup>®</sup>.

### Real-time PCR (qRT-PCR)

For statistical validation of the experiments, two independent tests in the thermal cycler Step OneTMAppliedBiosystems<sup>®</sup>, with mRNA extracted from the independent experiments, were performed. cDNAs of each experiment were used in quadruplicate for each condition evaluated. The medium for the PCR was prepared as follows (final concentrations): 5  $\mu\text{M}$  of the forward primer (Additional file 1: Table S1), 5  $\mu\text{M}$  of the reverse primer (1  $\mu\text{L}$ ) (Additional file 1: Table S1), 7.5  $\mu\text{L}$  of SYBR Green I component (Applied Biosystems<sup>®</sup>) and 0.5  $\mu\text{L}$  of ultra-pure water. 10  $\mu\text{L}$  of medium was added to an ELISA plate and 5  $\mu\text{L}$  of cDNA was added. For cDNA dilution curve, the following concentrations were used: 0.2, 2, 20 and 200 ng of cDNA template at the control condition. The whole procedure was performed in a laminar flow using sterile materials that were RNase free. After the addition of the reagents, the plate was sealed with adhesive and centrifuged gently. The protocol used for the experiment consisted of four steps: (i) program denaturation (10 min at 95 °C); (ii) amplification program and quantification repeated 45 times (10 s at 95 °C; 5 s at 61 °C—for both genes [reference and target]; 5 s at 72 °C with a single fluorescence acquisition mode); (iii) melting curve program (65–95 °C with a heating rate of 0.1 °C per s with continuum fluorescence acquisition) and (iv) cooling program to lower the temperature to 40 °C. Crossing points (CPs) were obtained and used in the subsequent calculations. CPs are defined as the point at which the fluorescence achieves significantly higher levels than non-specific fluorescence.

The relative mRNA expression of the interest genes and the endogenous control (ubiquitin [UBI]) (Additional file 1) were compared using a nonparametric pairwise fixed reallocation randomization test<sup>®</sup> as previously described [38]. The formula used to calculate the relative expression is as follows:

$$R = \frac{(E_{\text{target}})\Delta\text{CP}_{\text{target}}(\text{Control average} - \text{sample average})}{(E_{\text{ref}})\Delta\text{CP}_{\text{ref}}(\text{Control average} - \text{sample average})}$$

The equation represents the mathematical model used to calculate the ratio of relative expression in RT-qPCR. The ratio R of a target gene for a specific condition (sample or treatment) is presented versus the control condition compared to the reference gene (ref).  $E_{\text{target}}$  is the efficiency of RT-qPCR of the reference gene transcript.  $\Delta\text{CP}_{\text{target}}$  is the difference between the CP of the target genes transcripts at the control condition and the sample or treatment condition.  $\Delta\text{CP}_{\text{ref}}$  is the difference between the CP of the reference gene transcript at the control condition and the sample or treatment condition.

#### Measurements of H<sup>+</sup> fluxes using the ion-selective vibrating probe system

Extracellular H<sup>+</sup> fluxes were measured at the elongation zone of the main root of maize, treated or not with HA or PGPB, using an ion-selective vibrating probe technique [39–41]. Maize seedlings were placed in plastic Petri dishes (140 × 140 mm) filled with 20 mL of low salt-adapted nutrient medium composed by: 0.037 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM KCl, 0.1 mM MgSO<sub>4</sub>, 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 mL L<sup>-1</sup> Clark micronutrients, 0.025 mL L<sup>-1</sup> Fe-EDTA, pH 5.5. Ion-specific vibrating microelectrodes were produced as described by Feijó et al. [39]. Micropipettes were pulled from 1.5 mm borosilicate glass capillaries (<http://www.sutter.com>) using the Puller Flaming Brown, Sutter P-98 (Sutter Instruments, Novato, CA) and treated with *N,N*-dimethyltrimethylsilamina (C<sub>5</sub>H<sub>15</sub>NSi, Fluka 41716). After silanization, they were backfilled with a 15–20 mm column of electrolyte (15 mM KCl and 40 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.0) and then frontloaded with a 20–25 μm column of H<sup>+</sup>-selective liquid exchange cocktail (Fluka, Milwaukee, WI, USA). An Ag/AgCl wire electrode holder (World Precision Instruments, Sarasota, FL, USA) was inserted into the back of the microelectrode and established electrical contact with the bathing solution. The ground electrode was a dry reference (DRI-REF-2, World Precision Instruments) that was inserted into the sample bath. Signals were measured using an amplifier (<http://www.applicableelectronics.com>), while the vibration and the electrode positioning were obtained through motors (stepper motors), allowing

a three-dimensional movement. The control of the motors, data acquisition and its primary processing were adjusted through the ASET 2.0 software (Science Wares [East Falmouth, MA]—<http://www.sciencewares.com>). The calibration of the electrodes was conducted by measuring the potential (mV) recorded in three solutions containing the ion studied, with known concentrations: 0.1 mM, 1 mM and 10 mM, given that concentrations cover the conditions of the means used. The collection of data from the ion-selective vibrating probe, performed through the ASET software, provided the necessary information to calculate the ionic flow at a given point [x, y, z] of space, through Fick's law ( $J = D (dc/dx)$ ). The diffusion coefficient (D) is a standard value for each ion (in accordance with Handbook of Chemistry and Physics, Chemical Rubber Co.). The spatial difference (dx) resulted from the calculation of the distance between the two points that were made the measurements of concentrations to calculate the flow (15 μm). The concentration difference (cd) is a vector that varies during the test. At each point, the concentration can be calculated from the mV value recorded and the predetermined equation for the ionophore during the calibration process.

#### Statistical analysis

The experiment was arranged in randomized block designs with four biological replicates. The results were statistically analyzed by one-way ANOVA combined with Tukey's test at  $P < 0.05$ , using the program GraphPad Prism 7.0.

## Results

#### Humic acid (HA) characteristics

The HA isolated from sugarcane filter cake alone or in conjunction with *H. seropedicae* exhibited low carbon (46%), high nitrogen (5.7%) and high oxygen content (45%) compared with HA from pedogenic sources. As expected, the chemical nature of HA was characterized by an increase in carbohydrate moieties as revealed by CPMAS <sup>13</sup>C NMR spectrum (Additional file 2: Figure S1). The main signals present in the spectrum were a broad signal around 30 ppm due to CH<sub>3</sub> and CH<sub>2</sub> groups and two sharp peaks at 56 ppm and 72 ppm, which can be attributed to methoxy and O-alkyl groups, respectively. The broad resonance between 120 and 152 ppm is typical of aromatic and olefinic carbons, while the intense signal at 174 ppm reveals a large quantity of carboxyl groups compatible with high oxygen content as revealed by elemental composition analysis.

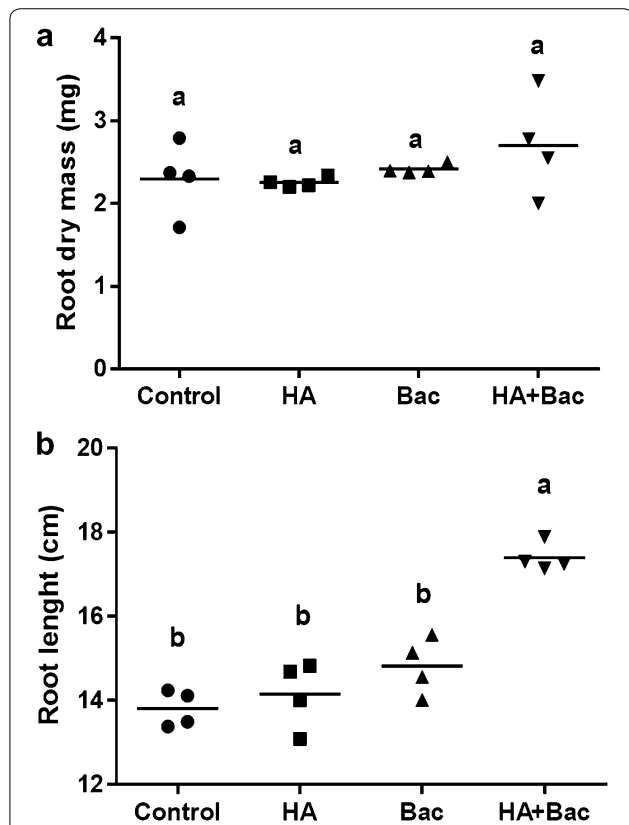


### Dry mass and root length

There was no significant difference in the root dry mass among the treatments (Fig. 1a). However, root length was stimulated by HA + Bac when compared to control plants (Fig. 1b). There was no significant difference in root length for the remaining treatments.

### Gene expression

The relative expression of *Mha1* was sixfold higher ( $P < 0.05$ ) for all treatments (HA, Bac and HA + Bac) with respect to the control (Fig. 2a). No statistical differences were observed among treatments (Fig. 2a). *ZmPIP1* encodes aquaporin 1 protein and functions in water transport. The relative transcription of this gene was six-, eight- and ninefold ( $P < 0.05$ ) higher in HA, Bac and Bac + HA treatments, respectively, when compared to the control (Fig. 2b). Repressed transcription of genes involved in  $\text{NO}_3^-$  transport was observed in all treatments (Fig. 3a, b), but was higher when HA or Bac were used singly than when the two treatments were combined (HA + Bac) (Fig. 3a, b).

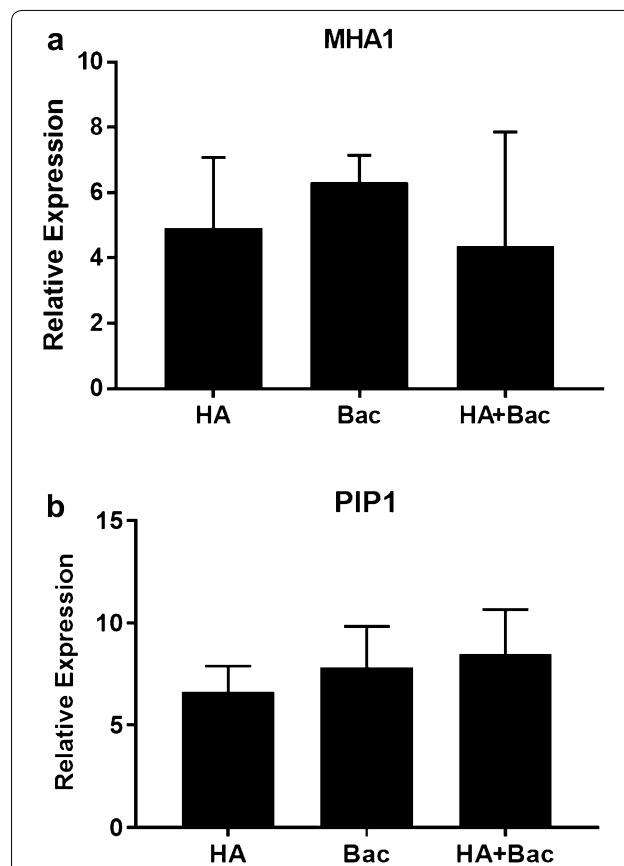


**Fig. 1** a Dry mass and b length of maize root seedlings treated with 50 mg C L<sup>-1</sup> humic acid (HA), *Herbaspirillum seropedicae* (Bac) or 50 mg C L<sup>-1</sup> humic acid and *H. seropedicae* (HA + Bac) for 7 days in hydroponic culture. Data were analyzed by one-way ANOVA combined with Tukey's test at  $P < 0.05$  ( $n = 4$ )

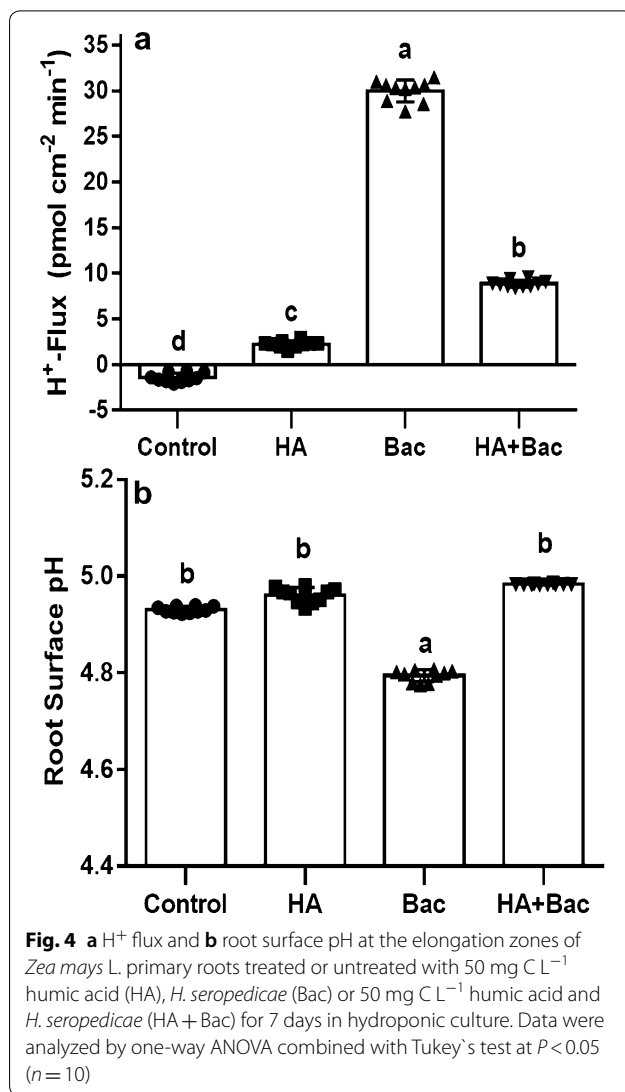
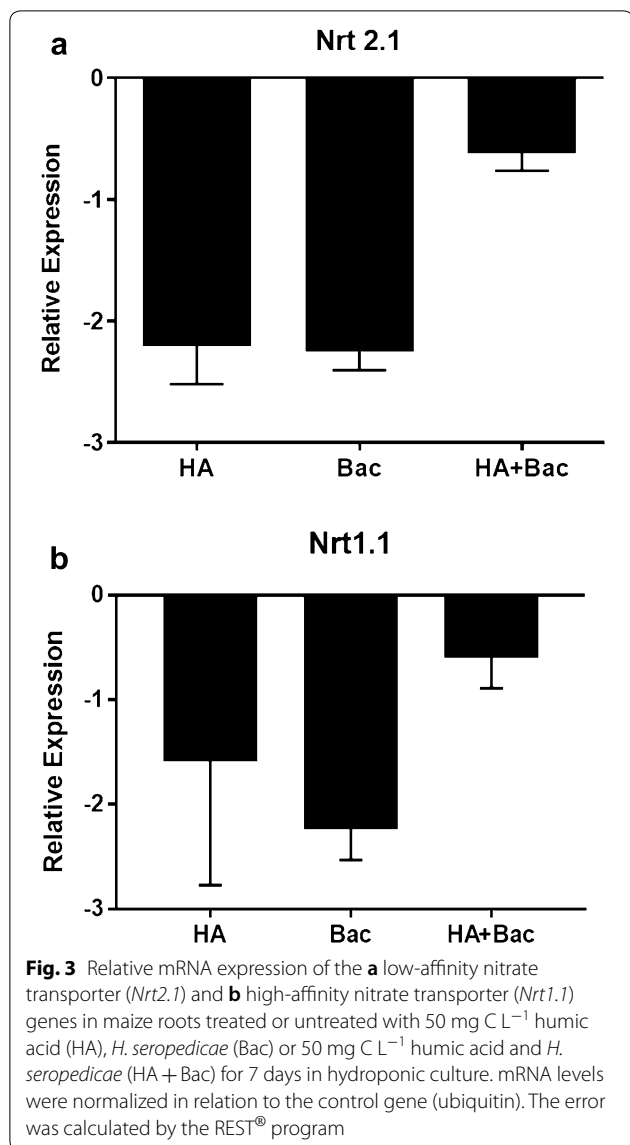
### Proton flux, root surface pH and voltage differences

The  $\text{H}^+$  flux at the elongation zone of maize roots was stimulated in all treatments when compared to the control; the highest flux was observed in the Bac treatment, followed by HA + Bac and HA only (Fig. 4a). An  $\text{H}^+$  influx of  $-2.0$  pmol was observed at the elongation zone of control plants (Fig. 4b). Roots treated with HA exhibited an  $\text{H}^+$  efflux of 3.5 pmol, and roots treated with Bac presented an even higher efflux of 25.0 pmol (Fig. 4a). In contrast, the combined treatment of HA + Bac provoked a reduction in the efflux of roots, with an average value of 12.0 pmol.

Changes in root surface pH correlated with  $\text{H}^+$  flux measurements. Significant differences were observed in the Bac treatment with lower pH (4.8) values; no significant differences were detected in the pH values of other treatments relative to the control (Fig. 4b). All treatments had positive voltage values, whereas the control treatment presented positive and negative  $\text{H}^+$  voltage values (Fig. 5). Plants treated with Bac exhibited the highest  $\text{H}^+$



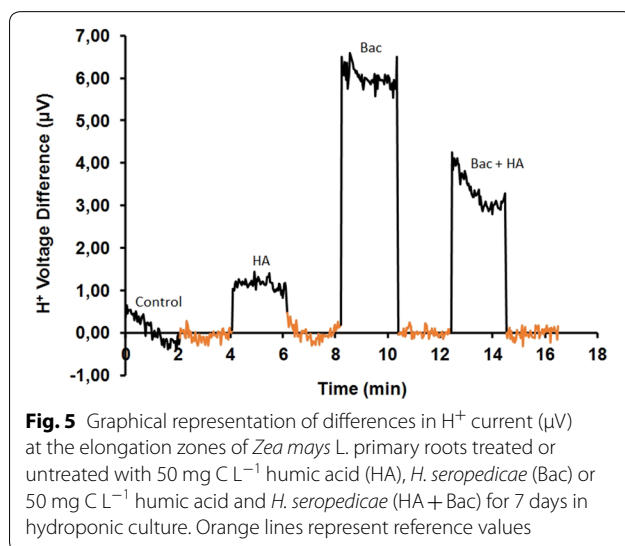
**Fig. 2** Relative mRNA expression of the a  $\text{P-H}^+$ -ATPase (*Mha1*) and b aquaporin (*ZmPIP1*) genes in maize roots treated or untreated with 50 mg C L<sup>-1</sup> humic acid (HA), *H. seropedicae* (Bac) or 50 mg C L<sup>-1</sup> humic acid and *H. seropedicae* (HA + Bac) for 7 days in hydroponic culture. mRNA levels were normalized in relation to the control gene (ubiquitin). Errors were calculated by the REST<sup>®</sup> program



voltage increase (mean of 6.0 μV), followed by HA + Bac and HA, with mean values of 3.5 and 1.0 μV, respectively (Fig. 5).

**Discussion**

Inoculants containing PGPB are important tools in overcoming problems associated with the excessive use of chemical fertilizers and pesticides [42]. In Brazil, the use of PGPB in non-leguminous plants remains restricted in comparison with soybeans. The co-inoculation of PGPB and HA resulted in increased yields for maize, sugarcane, pineapple, common bean and tomatoes [32]. The broad use of this low cost, environmentally friendly technology is partly dependent on understanding how the inoculants function in plant physiology. Here we describe the effect



of *H. seropedicae* (Bac) and HA in H<sup>+</sup> fluxes and membrane transporters.

The relative expression of Mha1 in maize seedlings treated with HA, Bac or HA + Bac showed increased expression and was up to sixfold higher than control plants. Façanha et al. [43] previously showed that solutions containing HA increased the number of P-H<sup>+</sup>-ATPases isolated from mono- and dicotyledonous roots. P-H<sup>+</sup>-ATPases play a central role in plant metabolism, because their activity generates an electrochemical gradient in cell membranes; these gradients are required for the activation of ion translocation, which is fundamental for the absorption of macro- and micronutrients [44]. In addition, the activity of P-H<sup>+</sup>-ATPases results in acidification of the apoplast and promotes increased cell wall plasticity, which facilitates growth and division of plant cells [45]. Canellas et al. [46] verified that the regulation of P-H<sup>+</sup>-ATPase synthesis by HAs isolated from vermicompost is typical of functional auxinic clusters that were detected in their structure. HS isolated from vermicompost regulates the expression of Mha1 and Mha2, which are involved in the synthesis of P-H<sup>+</sup>-ATPase in a manner similar to auxins [47] and are responsible for the induction of lateral roots and the synthesis, activation and expression of P-H<sup>+</sup>-ATPases [48].

Water channel proteins known as the MIP (major intrinsic protein) family have been identified in most living organisms. In plants, MIP was first identified in bean and represented approximately 2% of total proteins extracted from cotyledons [49]. A water channel function was first demonstrated in plants for a protein isolated from *Arabidopsis thaliana*, opening new perspectives in understanding mechanisms that regulate the transmembrane flow of water [50]. In this work, all treatments (HA, Bac or HA + Bac) promoted increased expression of the aquaporin gene, *ZmPIP1*. Furthermore, we demonstrated the contribution of *PIP1* and *PIP2* in water transport within roots, especially during conditions of water deficit [51, 52]. A prior work demonstrated that the co-inoculation of HA and PGPB enhanced water stress recovery, and inoculated sugarcane and beans exhibited a larger water content in leaves than untreated plants [53, 54]. The function of *PIP2* in the osmotic balance of *A. thaliana* root xylem cells was previously demonstrated by the analysis of T-DNA insertion mutagenesis [55]. Furthermore, an increase in aquaporin gene expression during N deficiency was observed by Liu et al. [56] and during salt stress when barley was inoculated with the nitrogen-fixing bacteria, *Azospirillum brasilense* [57]. The effect of HA in the differential expression of aquaporins in rice was previously described by Garcia et al. [58, 59].

The two main components directly involved in the uptake of NO<sub>3</sub><sup>-</sup> are P-H<sup>+</sup>-ATPases and NO<sub>3</sub><sup>-</sup> transporters. These components need to be coordinated with the availability of NO<sub>3</sub><sup>-</sup> in the medium for more efficient absorption. The transcriptional response of plants to the differential availability of NO<sub>3</sub><sup>-</sup> may be a key mechanism for survival in nutrient-poor environments; this could occur by differentially regulating specific genes for nutrient absorption, such as NO<sub>3</sub><sup>-</sup> transporters and P-H<sup>+</sup>-ATPase [60, 61]. We confirmed the transcriptional repression of NO<sub>3</sub><sup>-</sup> transport genes in all treatments (HA, Bac or HA + Bac). Increased expression of these genes was observed only in response to NO<sub>3</sub><sup>-</sup> fertilization [47]; this demonstrated that low MW humic fractions indirectly stimulate NO<sub>3</sub><sup>-</sup> uptake, possibly by increasing levels of Mha1 and Mha2 and inducing H<sup>+</sup> fluxes in the rhizosphere.

We observed increased H<sup>+</sup> flux in the elongation zone of maize roots treated with HA, Bac and HA + Bac. The increased H<sup>+</sup> flux at the elongation zone of roots inoculated with *H. seropedicae* is likely due to the activation of P-type H<sup>+</sup>-ATPases in response to auxins secreted by the bacteria. Previous reports suggest that many of the benefits provided by *H. seropedicae* are linked to auxin production [62]. Auxin secretion by bacteria can result in increased root volume, higher numbers of lateral roots and a larger capacity for nutrient uptake, thus resulting in greater promotion of plant growth [27, 63, 64]. Increased amino acid production, which may be attributed to the induction of H<sup>+</sup>-ATPases already present in the cell [65, 66], indicate a form of post-transcriptional regulation.

Previously, an increase in H<sup>+</sup> flux was reported for seedling roots treated with HA that was isolated from vermicompost [67]; this was associated with higher hydrolytic activity and H<sup>+</sup> transport of P-H<sup>+</sup>-ATPases, which then promoted a higher emergence of lateral roots in rice seedlings. Along with the increased of H<sup>+</sup> flux in plants treated with HA and HA + Bac, we predict an increase in the acidification of roots. This is likely because the P-H<sup>+</sup>-ATPase has a fundamental role in plant metabolism due to the activation of secondary transporters, acidification of the apoplast and nutrient absorption [68, 69]. Fluctuations in H<sup>+</sup> efflux have been described with biochemical and molecular changes involving the P-H<sup>+</sup>-ATPases, thus indicating that the bioactivity of HA impacts these enzymes in the plants [7, 26, 70]. The activity induced by HA is regulated partially by auxin, other plant hormones, various compounds, and functional groups in HA [4]. In this study, we show that the use of *H. seropedicae* singly or in combination with HA modified the root seedling H<sup>+</sup> flux and membrane voltage similarly but with different levels of magnitude;

this is logical since *H. seropedicae* reportedly induced the synthesis of auxin-like compounds and changed the hormonal balance in plants [36].

## Conclusion

The use of HA, Bac or HA + Bac altered the membrane potential of maize root cells, changing the electrochemical potential generated by P-H<sup>+</sup>-ATPase at both biochemical and molecular levels. We also observed changes in the expression of water transporters, thus revealing the physiological role of the combined inoculation of HA + Bac. The increase of H<sup>+</sup> efflux and cellular water content is pivotal in efforts to enhance nutrient and water efficiency. Drought conditions are predicted to increase with climate change [71], along with problems due to the high cost and environmental impact of nitrogen fertilizers. The inoculation of plants with HA + Bac induces physiological changes that can help plants sustain the impact of reduced nitrogen and drought stress.

## Additional files

**Additional file 1: Table S1.** Primer sequence of the genes analyzed. Ubiquitin (Ubi), aquaporin (Pip), low-affinity nitrate transporter (Nrt2.1), high-affinity nitrate transporter (Nrt1.1), P-H<sup>+</sup>-ATPase (Mha1). F = Forward, R = Reverse.

**Additional file 2: Figure S1.** CP-MAS <sup>13</sup>C NMR spectrum of humic acids used in the experiments.

## Abbreviations

HA: Humic acids; Bac: Cell suspension of *Herbaspirillum seropedicae*; HA + Bac: Humic acids and *Herbaspirillum seropedicae*; HS: Humic substances; MIP: Major intrinsic proteins; Ubi: Ubiquitin; PIP: Aquaporin; Nrt2.1: Low-affinity nitrate transporter; Nrt1.1: High-affinity nitrate transporter; Mha1: PM-H<sup>+</sup>-ATPase; NO<sub>3</sub><sup>-</sup>: Nitrate; PGPB: Plant growth-promoting bacteria; PM-H<sup>+</sup>-ATPase: Plasma membrane proton ATPase.

## Authors' contributions

IGA carried out the experiments and wrote the first version of this paper; FLO and LPC contributed to the experimental idea. ACR and AAB were responsible for the ion-selective vibrating probe system analysis. All authors read and approved the final manuscript.

## Author details

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## Competing interests

The authors declare that they have no competing interests.

## Consent for publication

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

## Ethics approval and consent to participate

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