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Plant chemical priming by humic acids

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Abstract

Background: Global market of humic substances has been increasing steadily based on the perception of the multifunctional properties as plant biostimulant, microbial vehicle and plant protective agent against environmental stress. Some field assays and many experimental observations have shown that humic matter could relieve the abiotic stress effects. Here, we explored the plant chemical priming effect concept, i.e., plant preconditioning by prior exposure to an appropriate dose of humic acids with the objective to reduce toxicity from a subsequent harmful exposure to abiotic stressor, such as salinity, drought, heavy metals and humic acids themselves.

Materials and methods: The prime state (PS) was characterized using traditional stress markers like proline content and catalase activity was well as the transcription level of mRNA of phytohormones-responsive genes, cell signaling, stress-responsive genes and transcription factors. A dose–response curve was built for stressor agents since maize seedlings in the PS were submitted to salinity, drought, chromium toxicity and humic acids concentration to reduce 50% of root fresh weight with respect to control plants.

Results: The PS or adaptive response by biostimulation of humic substances was described at transcriptional level, where the hormonal signaling pathways including abscisic acid, gibberellic and auxins, specific abiotic functional and regulatory stress-responsive genes were positively modulated. The negative impact of stressor agents was alleviated in the maize seedlings primed by humic acids.

Conclusion: Chemical priming by humic substances is a promising field tool in plant stress physiology and crop stress management.

Keywords: Abiotic stress, Stress alleviation, Biostimulant, Sustainable agriculture, Hormesis

Introduction

The importance of improving organic matter contents in agricultural soils is a consensus due its influence on soil properties and plant growth [1]. The effect of soil organic matter loss is more pronounced in tropical zone where modern industrial agriculture has been resulted in high productivity due to input intensification. However, economic, social and environmental costs are very high bringing risks to sustainability [2, 3]. Biological inputs can be used as suitable tool to help the transition for other agriculture systems [4].

Humic substances can be used directly on plants at low concentrations to enhance plant growth, yield and nutrient uptake, thus constituting a popular category of plant biostimulants [5]. One most intriguing aspect of direct use of humic substances on plants is how the complex and heterogeneous mixture of small organic molecules [6] can influence diverse physiology processes including nutrient uptake, proteome, metabolome and differential gene transcription [7–13].

The last data meta-analysis considering humic substances and plant growth reported an average increase of 20% in both shoot and root dry weight independent of plant type, humic source and concentration [14]. Moreover, the presence or absence of stress played a significant role in the data interpretation due its significant coefficient's weight [14].

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Table 1 shows a summary of common responses of different plants treated with humic substances against osmotic stress (salinity and drought) and heavy metal toxicity.

The effect of humic substances in the mitigation of abiotic stress effects in plants is well known and generally described as result of increase of enzymatic and non-enzymatic antioxidant defense, increase in compatible solutes production and changes in ion balance (see references cited in Table 1). During exposure to different abiotic stress, reactive oxygen species (ROS) are one of the major causes of cellular damage [45, 46]. To prevent ROS-induced oxidative injury antioxidative enzymes such as superoxide dismutase, peroxidases, catalase and ascorbate peroxidase and non-enzymatic production of scavenge compounds like ascorbate, tocopherol and phenolics are induced; compatible solutes such as proline are also produced to protect cells against ROS accumulation under stress conditions. All of these mechanisms are also triggered by humic substances (Table 1).

One of the most studied physiological effects of humic substances is the promotion of ion uptake mediated by the synthesis and functionality of membrane proteins, especially proton pumps that increase the electrochemical proton gradient across the plasma membrane [46–54]. Changes on ion balance [55] as well as exudation yield [56, 57] can be also included as part of general mechanisms against stress conditions promoted by humic substances.

The potential role of humic acids (HA) in preventing oxidative stress in plants was described by García and colleagues [43] that reported enhancement of peroxidase activity, reduction of H_2O_2 concentration and increase of cell proline levels leading to decreased reactive oxygen species (ROS) contents and thereby restoring the cytosolic redox homeostasis [58].

It was reported by Guridi-Izquierdo and collaborators [59] that seedlings previously treated with HA endured better the osmotic stress induced by polyethylene glycol (PEG 6000). Sugarcane plants previously treated with HA also recovered better from drought induced by omission of irrigation, and after the rehydration period an increase of antioxidant enzymatic activity (catalase, superoxide dismutase, and glutathione reductase and ascorbate peroxidase) and significant changes in metabolic profile [13] were observed.

Tomato seedlings pre-treated with a leachate from vermicompost were more efficient in mitigating the salinity damage providing a recorded great osmotic adjustment, with maintenance of net photosynthesis and K^+/Na^+, highest proline content in leaves and the highest sugar content in roots [19]. The use of HA for plant biostimulation aiming to revegetate areas contaminated with heavy metals was proposed [59]. All of these reports hold in common the concept of preconditioning of plants by humic matter [13, 19, 44, 59, 60] to better withstand further stress exposition.

Plants can be 'prepared' (primed state or PS) to more successfully tolerate future biotic and abiotic stress conditions [61]. Chemical priming involves exposure to a priming agent such as a natural or synthetic chemical compound including amino acids, hormones, and reactive oxygen–nitrogen–sulfur species [61]. The use of humic substances as chemical prime agents was not yet considered, but the application of biostimulants has been reported as a tool for plant hormesis management [62]. These authors defined hormesis as a "*phenomenon by which a stressor* (*i.e., toxins, herbicides,* etc.*) stimulates the cellular stress response, including secondary metabolites production, in order to help organisms to establish adaptive responses*" [62]. It is well known that humic substances can enhance the plant secondary metabolism inducing phenyl alanine ammonia lyase activity and phenolic content [63, 64] modifying the shikimic acid pathway [13].

The objective of this work was to induce the PS of maize seedlings by HA with the aim to alleviate further symptoms of stressors (salinity, drought, heavy metal toxicity and HA itself). The cell signaling components, transcription factors and some gene stress response were monitored by transcriptomic analysis.

Materials and methods

Humic acids-like isolation and characterization

A solution of 0.5 M NaOH was mixed with earthworm compost (10:1, v/v) under a N_2 atmosphere. After 12 h, the suspension was centrifuged at 5000×*g* and the humic acids (HA) were precipitated by adding 6 M HCl until pH 1.5. After centrifugation (5000×*g*) for 15 min, the sample was repeatedly washed with water until a negative test against $AgNO_3$ was obtained. Subsequently, the sample was dialyzed against deionized water using a 1000-Da cut-off membrane (Thomas Scientific, Swedesboro, NJ, USA) and lyophilized. The HA solution was prepared by solubilizing HA powder in 1 mL of 0.01 M NaOH, followed by pH adjustment to 6.5 with 0.1 M HCl. 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 assessed 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

Table 1 Common mechanisms related to symptom relief of different types of abiotic stress (salinity, drought and heavy metal toxicity) by humic substances applied directly to plants or soils

Salinity				
Osmoprotection/osmotic adjustment	**Antioxidant activity**	**Ionic balance**	**Water efficiency use**	**References**
Proline; sugars, phenols	–	✓	–	[15]
Proline, sugars,	CAT, SOD; ascorbic acids	✓	✓	[16]
Soluble protein	CAT, POX	✓	–	[17]
Soluble sugars, proline	CAT, SOD, GPOX; ascorbic acid, glut, TOC	✓		[18]
Soluble sugars		✓	–	[19]
Pigments	Ascorbic acid, phenols, anthocyanins	✓	✓ RWC	[20]
Proline	SOD, CAT, POX	–	–	[21]
–	–	✓		[22]
Pigments	–	✓		[23]
MDA, proline, H_2O_2	SOD, POD, CAT	✓	✓	[24]
–	–	✓		[25]
MDA. H_2O_2	SOD, APX, GST			[26]
Proline, free sugars		✓	✓	[27]

Heavy metal stress				
Stress marker	**Antioxidant activity**	**Uptake**	**Toxicity**	**References**
Proline	CAT, SOD, POX	–	Fe	[28]
	CAT, SOD, POX	–	Pb	[29]
	SOD, CAT	+	Cd	[30]
	SOD, POX	–	Cd	[31]
Protective DNA activity			Mn	[32]
		+ or − according HA concentration	Cd	[33]
Decrease on H_2O_2 production and lipid peroxidation		–	Pb	[34]
Proline. MAD	SOD, CAT, GPOX, GST		Cu, Cd	[35]
		–	Pb, Cd	[36]
Sugars, soluble proteins RWC		–	Cd	[37]
		Decrease	Cd	[38]

Drought			
Osmoprotection/osmotic adjustment	**Antioxidant activity**	**Water efficiency use**	**References**
–	–	Improve net photosynthesis	[39]
Changes in metabolic profile	CAT, POX and SOD	RWC	[40]
Proline, vitamin C	CAT, POX	RWC and (WEU)	[41]
Decrease of MDA; proline	Enhancement of CAT, POX, APX		[42]
MDA	Enzymatic: POX; decrease of H_2O_2 content	Enhance of aquaporins gene expression (TIPS)	[43]
Proline,	Enzymatic: POX; decrease of H_2O_2 content		[44]

CAT: catalase activity; SOD: superoxide dismutase activity; POX: peroxidases; APX: H_2O_2: hydrogen peroxide concentration; RWC: relative water content; WUE: water use efficiency; GPOX: GST: MAD: malondialdehyde heavy metals: Cu (copper) Cd (cadmium): Fe (iron): Pb: Mn

chemical shift (ppm) resonance intervals of 0 to 46 ppm (alkyl C, mainly CH_2 and CH_3 sp^3 carbons), 46 to 65 ppm (methoxy and N alkyl C from OCH_3, C–N, and complex aliphatic carbons), 65 to 90 ppm (O-alkyl C, such as alcohols and ethers), 90 to 108 ppm (anomeric carbons in carbohydrate-like structures), 108 to 145 ppm (phenolic carbons), 145 to 160 ppm (aromatic and olefinic sp^2 carbons), 160 to 185 ppm (carboxyl, amides, and esters), and 185 to 225 ppm (carbonyls).

Dose–response curve (HA, NaCl, PEG 6000, Cr_2O_7)

Maize seeds (*Zea mays* L., var. Dekalb 177) were surface-sterilized by soaking in 0.5% NaClO for 30 min, followed by rinsing and then soaking in water for 6 h. Afterward, the seeds were sown in 2.0-L Leonard's pots filled with washed and sterilized sand wetted with 1/3 strength Furlani nutrient solution (μmol L^{-1}: 3.527 Ca; 2.310 K; 855 Mg; 45 P; 587 S; 25 B; 77 Fe; 9.1 Mn; 0.63 Cu; 0.83 Mo; 2.29 Zn; 1.74 Na; and 75 EDTA) with the N content adjusted to a low concentration (100 μmol L^{-1} $NO_3 + NH_4$). Six replicates were used in a randomized statistical design. After 1 week, the solution was changed for one-half of the ionic force. At 7 days after planting, the maize seedlings were submitted to treatments: humic acids diluted with low N Furlani nutrient solution at 0, 0,1, 1, 10, 100 and 1000 mg L^{-1} : salinity: NaCl 0, 30, 60, 90 and 120 mM; drought: PEG 6000 0, −0.40, −0.60, −0.80 and −1.20 MPa; heavy metal toxicity: $K_2Cr_2O_7$ 0, 1, 10, 100 and 1000 ppm. Six maize plants were collected at 14 days after treatment for growth analysis. The fresh weight of roots and shoots were measured.

Characterization of plant prime state (PS) induced by HA

The root extracts of maize seedlings treated with different HA concentration were obtained at 4 °C. Plant tissues (1 g fresh weight) were homogenized in 10 mL of 100 mM EDTA, 3 mM DTT and 4% (w/v) PVP. The homogenate was filtered through five layers of cheesecloth and centrifuged at 17,000*g* for 30 min. The supernatant was collected, and aliquots were frozen at −70 °C until analysis of catalase (CAT) activity and proline content. The CAT activity was assayed spectrophotometrically at 25 °C in a reaction mixture containing l mL 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 μL H_2O_2 (30% solution) prepared immediately before use. The reaction was initiated by adding 15 μL of plant extract and the activity was determined by following the decomposition of H_2O_2 by the change in absorbance at 240 nm for 2 min against a H_2O_2-free blank. The content of free proline was determined after the reaction contained 2 mL of glacial acetic acid, 2 mL of ninhydrin reagent (2.50% w/v ninhydrin in 60% v/v 6 M phosphoric acid) and 2 mL of extract. The incubation lasted for 1 h at 90 °C. The upper toluene phase was decanted into glass cuvette and absorbance was measured at 520 nm. The concentration was assayed using proline as the calibration standard.

Transcriptional analysis of humic acid-treated maize root plants

For RNA extraction, 100 mg of control roots and HA-treated roots, using the best dose for root growth at 4 mM C HA L^{-1} was macerated in liquid nitrogen. The total RNA of the samples (3 biological replicates per treatment) was extracted with the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA was quantified using the Nanodrop 1000 spectrophotometer. The RNA was eluted in DEPC-treated water (total amount of 4–10 μg RNA) digested with DNAse and depleted of ribosomal RNA using the GOTAQ® 1-STEP RT-QPCR (PROMEGA). Subsequently, a 1% free RNAse agarose gel was made to analyze the RNA extracted. Sequencing libraries were prepared using the Whole Transcriptome Analysis kit (Applied Biosystem) according to the manufacturer's protocol. Libraries were sequenced on the Illumina platform by LacTad company—Brazil. To perform bioinformatics analysis of the sequences obtained by RNA-Seq, the reads obtained from the RNA-Seq were analyzed to identify ribosomal RNA (rRNA) sequences in two steps: (1) rRNA sequences of *Zea mays* were downloaded from NCBI and an index file of rRNA was created using Novoalign v3.06.05. (http://www.novocraft.com/products/novoalign/). Then reads were mapped on index file using Novoalign; (2) all fastq files were converted into Fasta and BLASTN analysis was performed against downloaded rRNA sequences. Identified rRNA sequences were removed and reads were cleaned. Further, quality of all reads was assessed by running the FastQC software [65] and high-quality cleaned reads were aligned on *Z. mays* genome using Novoalign. Gene expression levels were normalized as reads per kilobase of transcript per million mapped reads (RPKM). The differential gene expression between control and inoculated were determined by using Cuffdiff v2.2.1. The genes with differences of at least onefold change along with adjusted p value (FDR) ≤ 0.05 were considered to be significantly differentially expressed. Functional classification analysis was executed with MapMan version 3.6.0RC1 (https://mapman.gabipd.org/).

Characterization of symptoms of stressor agents in plant PS

Maize seedlings in PS induced by the best dose of HA were further exposed to abiotic stress (salinity, drought heavy metal toxicity and HA itself) by a rate of each abiotic stress agent that promoted the reduction of 50% of the root fresh weight. In the preconditioning experiment, we treated or not (control) maize seedlings with 100 mg HA for 7 days to induce plant PS. After this time, the seedlings were submitted to 60 mM NaCl, PEG6000 −0.4 MPa; 100 mg L^{-1} $K_2Cr_2O_7$ and 1000 mg L^{-1} HA and after 1 week the root fresh weight of seedlings were measured. The one-way ANOVA was performed using the program GraphPad Prism 7.0.

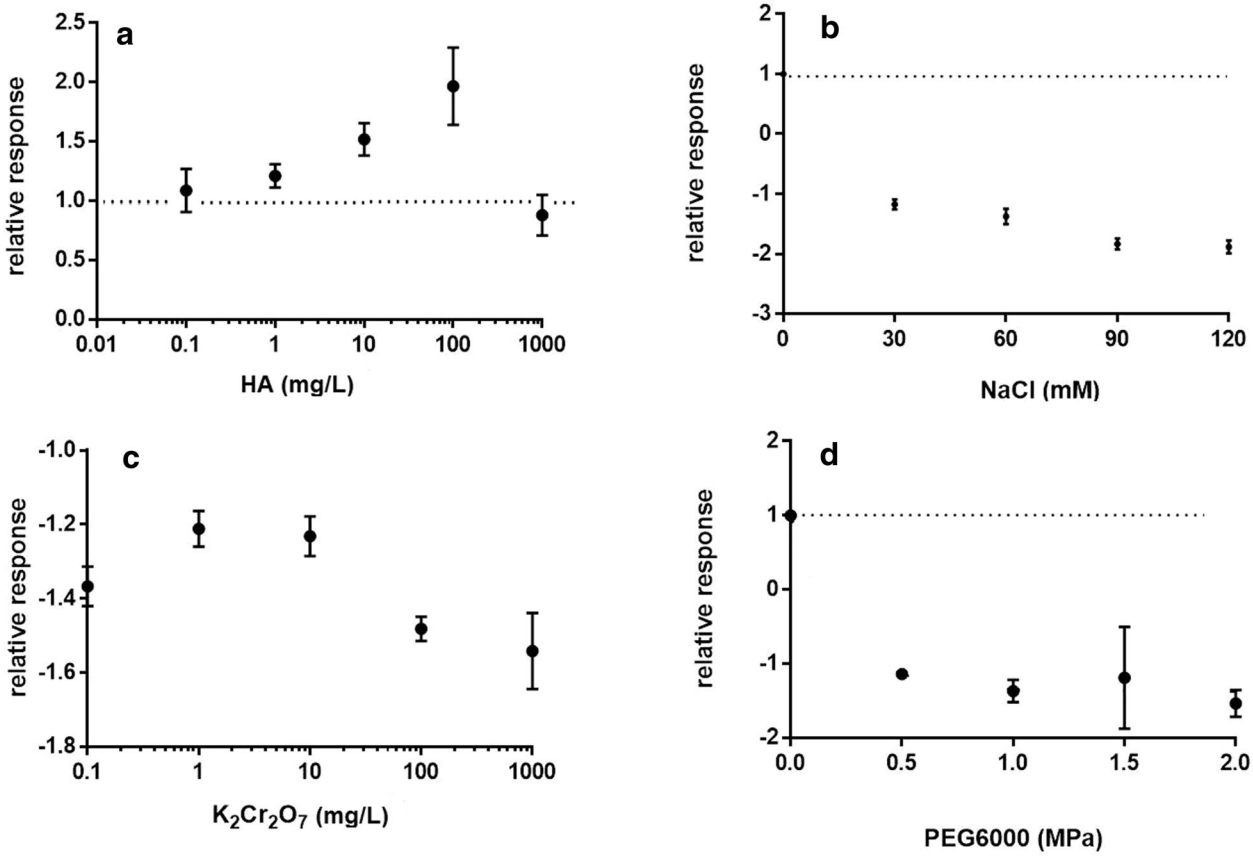

Fig. 1 Dose–response curves for effects of humic acids and three plant toxicants on root fresh weight of maize seedlings (data give means of 4 replicates per dose followed by standard deviation). **a** Humic acids isolated from vermicompost (HA); **b** sodium chloride (NaCl); **c** potassium dichromate ($K_2Cr_2O_7$); **d** polyethylene glycol (PEG MG 6000). Dashed lines represent the control response level

Results

Humic acids-like characteristics

The HA used in this study showed low carbon (47%), high nitrogen (6%) and oxygen content (44%). Its chemical nature was characterized by large carbohydrate moieties as revealed by CPMAS ^{13}C NMR spectrum (Additional file 1: S1). The main signals present in the spectrum were a broad signal around 30 ppm due to CH_3 and CH_2 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.

Dose–response curve of humic acids and stressor agents

The HA showed high bioactivity typical from vermicompost with bell-shaped dose–response curve (Fig. 1a) with best dose ranging from 3.5 to 4 mM C L^{-1}.

The root fresh weight at best HA concentration was around 80% higher that control in a typical stimulation range reported previously using HA isolate from vermicompost [51]. The root fresh weight of maize seedlings treated with different abiotic stress is also shown in Fig. 1, and so are the concentrations responsible for reduction of approximately 50% of roots fresh weight, which are 60 mM NaCl (Fig. 1b), 100 mg L^{-1} $K_2Cr_2O_7$ (Fig. 1c) and − 0.40 MPa PEG 6000 (Fig. 1d). The largest HA concentration (Fig. 1a) was used in further experiments.

Characterization of prime state of maize seedlings

The previous treatment with the best dose of HA induced the classical mechanisms of abiotic stress defense monitored by antioxidant enzymatic activity and compatible solutes accumulation. In the best HA dose, CAT activity was 20% larger with respect to control and proline content on roots was 18% larger (Fig. 2).

RNA-seq transcriptional analysis was performed for three independent biological replicates of maize root tissue of each treatment (control and HA-treated plants)

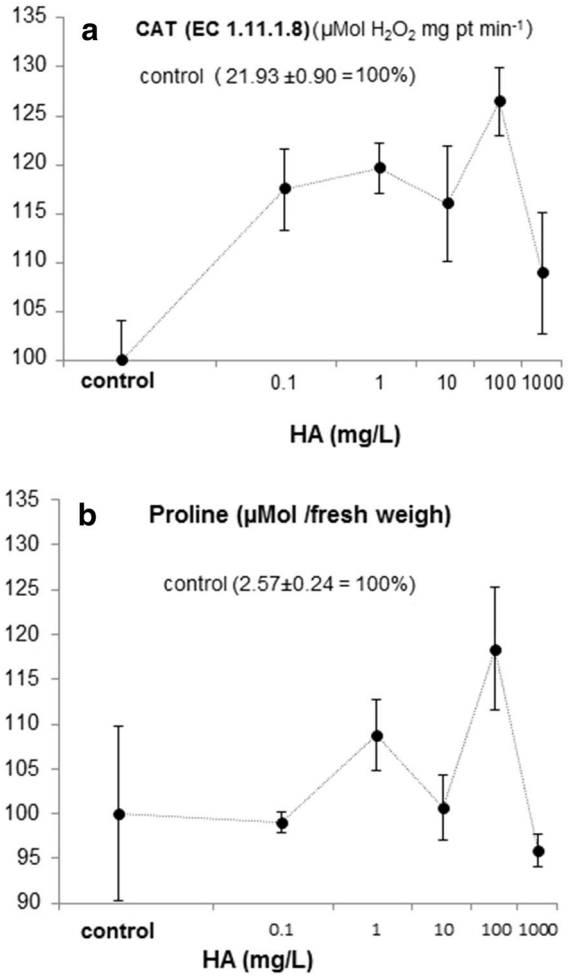

Fig. 2 Dose–response curves for effects of humic acids on maize seedlings stress markers: **a** catalase activity, **b** proline concentration

generating six libraries. Control and humic acid (HA) samples generated, respectively, 44.76 and 46.05 million data sequences and uniquely mapped read values presented for each sample (Table 2). The reads were mapped against *Z. mays* genome. Differential gene expression levels of HA-treated roots as presented as fold-differences in relation to control root plants with at least onefold change along with adjusted *p*-value (FDR) ≤ 0.05 were considered to be significantly differentially expressed. A general quantitative view of differential expressed genes related to main regulatory pathway such as transcription factors, protein dynamics, hormone responsive and cell signaling is seen at Fig. 3. Later on, we focused the transcriptomic analysis on RNA related to hormonal signaling and stress perception in the subsequent kinases transcription factors (TFs) and stress gene response.

We showed only the values of upregulated genes by HA with respect to control in the PS that were significant for the *t* test ($p < 0.05$). A wide range of auxin-induced proteins in larger transcriptome level including auxin-responsive family, auxin response factors and auxin signaling F box proteins (Fig. 4) were observed. The SAUR-like auxin responsive was the family transcribed in larger level. Other hormone-related genes were induced in the PS including ethylene-responsive element binding and gibberellin and brassinosteroids oxidases. As expected some genes encoding ABA signaling were induced by HA including ABA-responsive (TB2/DP1) and ABA-responsive element binding. Ca^{2+} is the well-known and reported secondary cell messenger involved in the stress perception. A larger number of Ca^{2+} receptors triggered by HA were observed.

Among them calmodulin, calmodulin-binding receptor like, calcium-binding EF family, calcineurin, calcium-dependent phospho-transpherase and calcium-dependent protein kinase (CDPK) were observed.

The core of signaling amplification is the phosphorylation reactions made by several kinases proteins besides CDPK. The transcription level of kinases in the PS state is shown in Fig. 5.

Protein kinases, protein serine/threonine kinases and phosphatases, protein phosphatases 2 A sub 2A, kinases associated to protein phosphatase and highly ABA-induced group-A protein phosphatases type 2C (PP2C) were found in larger level than in control (Fig. 6). These kinases/phosphatases are active in generic phosphorylation pathways. Many kinase proteins involved in multiple protein–protein interactions and assembly

Table 2 General RNA-seq mapping

Samples	Number of reads (total input)	Number of reads (in millions)	Number of mapped reads	Uniquely mapped reads (%)	Multiple mapped reads (%)	No mapped reads (%)
Control 1	15,093,794	15.09	10,688,849	55.91	14.9	29.2
Control 2	19,631,281	19.63	14,525,758	37.84	36.16	26.0
Control 3	19,038,784	10.04	13,777,386	61.99	10.38	27.6
Humic acid 1	16,098,633	16.10	11,116,446	56.46	12.6	30.9
Humic acid 2	16,855,415	16.86	11,825,087	61.06	9.1	29.8
Humic acid 3	13,087,821	13.09	8,996,167	58.53	10.21	31.3

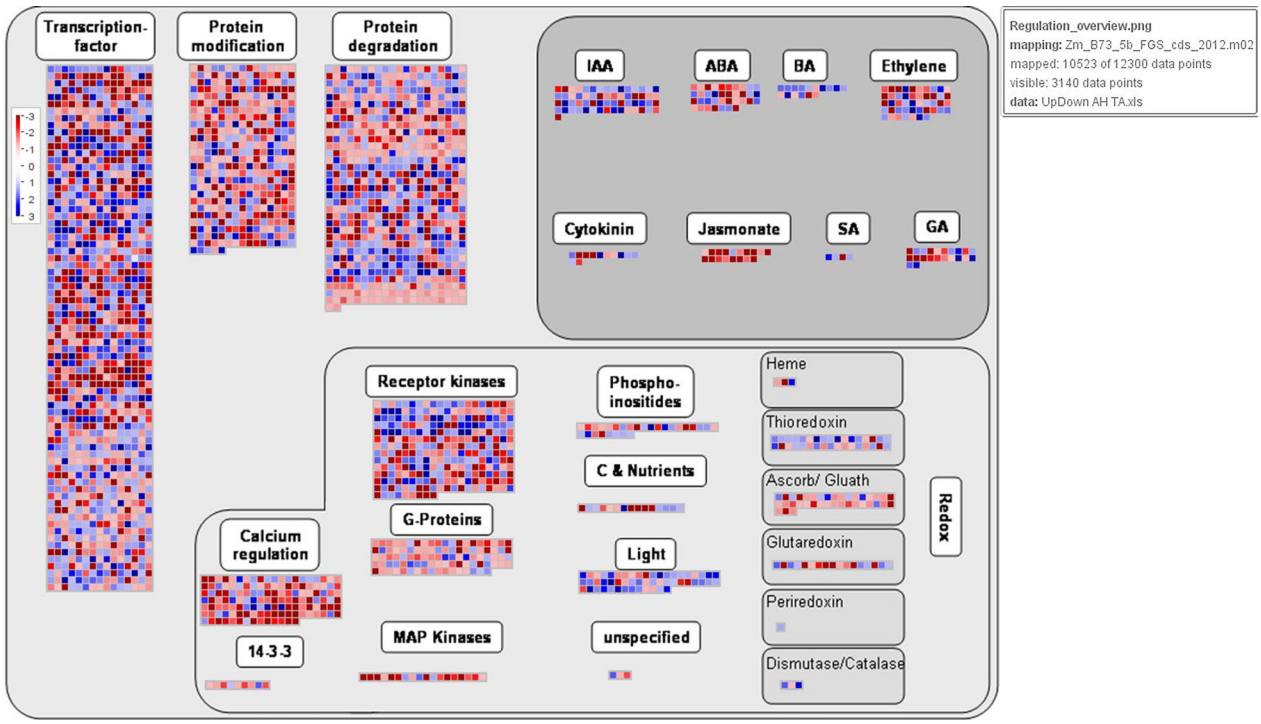

Fig. 3 MapMan functional classification analysis of differential expressed genes related to the plant cell regulatory pathways. Blue and red dots mean upregulated and down regulated genes when 4 mM C L^{-1} humic acid was applied to the maize roots

of multiprotein complex were observed in larger level including protein kinase tetratricopeptide repeat domain, leucine-rich domain, adenine nucleotide alpha hydrolases, octisapeptide/Phox, Bem1p domain. In addition, a high level of mitogen-activated protein kinases (mapk), a universal signal transduction module involved in responses to various biotic and abiotic stresses, hormones, cell division and developmental processes was also observed. PYR1 is a receptor for ABA required for ABA-mediated responses and responsible for inhibiting the activity of PP2Cs when activated by ABA and observed in high level of transcription.

Plant gene expression, in response to stress cues, is tightly controlled by transcriptional regulators. The main transcription factors (TF) related to abiotic stress response can be oversimplified in two categories: (i) ABA dependent including myeloblastosis oncogene (MYB) and myocytomatosis oncogene (MYC) regulon, ABA-responsive element binding protein (AREB) and ABA binding factor (ABF) and (ii) ABA-independent TF including NAC and zinc-finger homeodomain (ZF-HD) regulon. All of these TFs were induced in the preconditioning phase by HA in comparison with control (Fig. 6). It was also observed in the PS state induced by HA, i.e., without presence of abiotic agent the significant large transcriptional level of genes related to abiotic stress response, as well as proteins involved in the cell autophagy process (Fig. 7).

HA clearly triggered the priming stimulus resulting in abiotic stress tolerance.

Alleviation symptoms of further stressor agents' exposition

The subsequent exposition of maize seedlings primed by HA by different abiotic stress did not affect root growth (Fig. 8) with exception of chromium toxicity, the increase of which observed in the PS seedlings was not enough to significantly show a difference ($p < 0.05$).

PS seedlings showed greater shoot fresh weight than in stressed seedlings. One can see the data of shoot fresh weight in Additional file 2: S2 since the biochemical and transcription analyses were limited just to root tissues due to the economic limitations imposed by the current Brazilian government's suicidal scientific policy. We observed a clear biostimulation effect of HA concomitantly with the plant chemical priming against different abiotic stressors.

Discussion

The molecular characteristics of humic acids isolated from vermicompost revealed by ^{13}C-CP/MAS NMR spectrum is similar to those observed in other humic

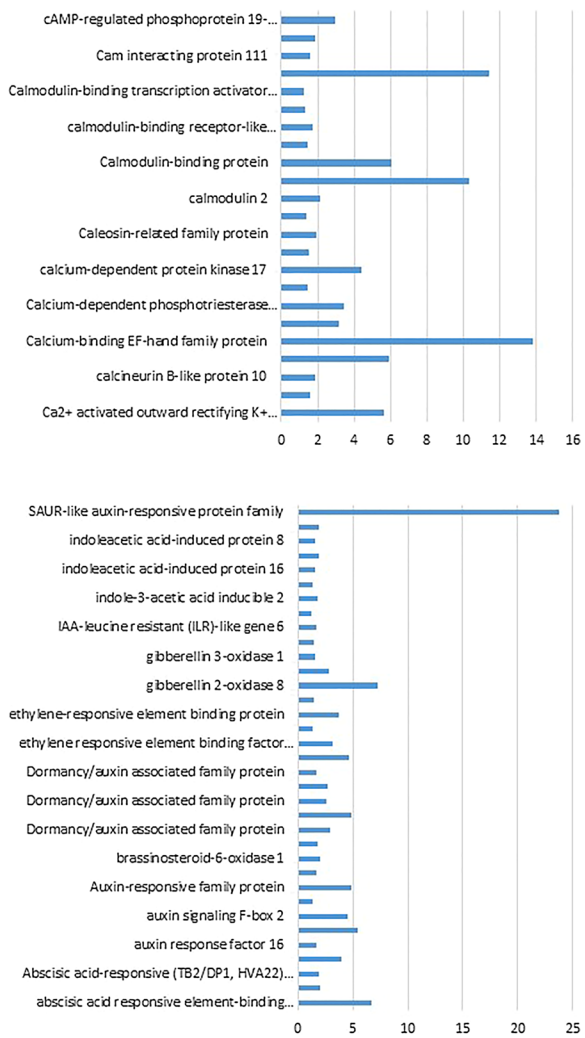

Fig. 4 Transcription level of genes encoding proteins related with Ca^{2+} and hormonal signaling. The values represent the means of three replicates and are with respect to control level = 0%. Only the significant values at $p < 0.05$ by *t* test are shown

acids with high bioactivity due to the presence of well-resolved signals at 56, 125, 150 and 175 ppm that were previously associated with induction of plasma membrane (PM) proton pumps and promotion of lateral root emergence in maize seedlings [66]. Vermicompost is a renewed source for extraction of humic substances with high biological activity and its plant growth promotion are closely linked with its chemical nature [67–69].

The concept of plant chemical primed by HA to alleviate subsequent abiotic stress effects was intuitively used in previous works [40, 43, 44, 59]. Here, we characterized the PS using the transcriptome approach (Figs. 4, 5, 6 and 7) and CAT activity and proline concentration as abiotic stress marker (Fig. 3). We found a clear and typical response against abiotic stress in the PS induced by HA. Previously, it was observed that HA can promote the ROS scavenge through ABA-independent mechanisms [44] and plant secondary metabolism activation, including the enhancement of phenolics content [64]. Both mechanisms are typical of actual plant defense priming [70]. It is a clear manifestation of hormesis phenomena defined previously in the introduction [61, 62]. As no match was found in the web using "humic" and "hormesis" as keyword, we reintroduce the concept in order to discuss it in the crop stress management context. The first paragraph of Calabrese´s review [71] summarizes the PS indicating that *preconditioning in the biological and biomedical sciences is a phenomenon in which a prior exposure to an appropriate low dose of a toxic agent or stress reduces toxicity from a subsequent harmful exposure* (*i.e., challenging dose*) *of the same, a related, or an unrelated toxic/stressor agent.* In this review, one can see when and how the term was used in the medical science including the post conditioning effect. The adaptive phenomena in general and chemical-induced adaptive response can be considered as specific manifestations of hormesis, i.e., a biphasic dose–response phenomena [72].

The same quantitative features to hormetic dose responses for animals were found for plant extracts [73]. This can be a keystone theory to justify the plethoric effect of HA on plant physiology. The traditional view (macromolecular/polymeric) of humic substances was overcome from Prof Alessandro Piccolo's work (for an update on the process of humification, composition and structural arrangement see reference [6]). Now, the question raised by the humeomic approach [74] is whether the bioactivity is the result of each small and heterogeneous molecule present in the humic matrix or is it an emergent property of the all "humic extract"? The priming plant mechanisms can provide a plausible explanation for the plethoric effect of humic substances in plants. In addition, other benefits induced by HA should be considered, such as the low fitness and ecological costs, robust defense and better plant performance.

PS of seedlings induced by HA enhanced defense against diverse abiotic stress, and (i) we characterized the PS induced by HA at transcriptional level, and (ii) we observed the mitigation of abiotic stress symptoms in further exposition to salinity, drought, chromium toxicity and high HA concentration (Fig. 8). The transcriptomic analysis allowed to assess the PS and understand how it is possible to use HA at low concentration to mitigate the different abiotic stress, without presence of stressors. At the highest transcriptional level, diverse genes were found to encode the main plant hormones receptors, as well as cell signaling, stress perception, kinases

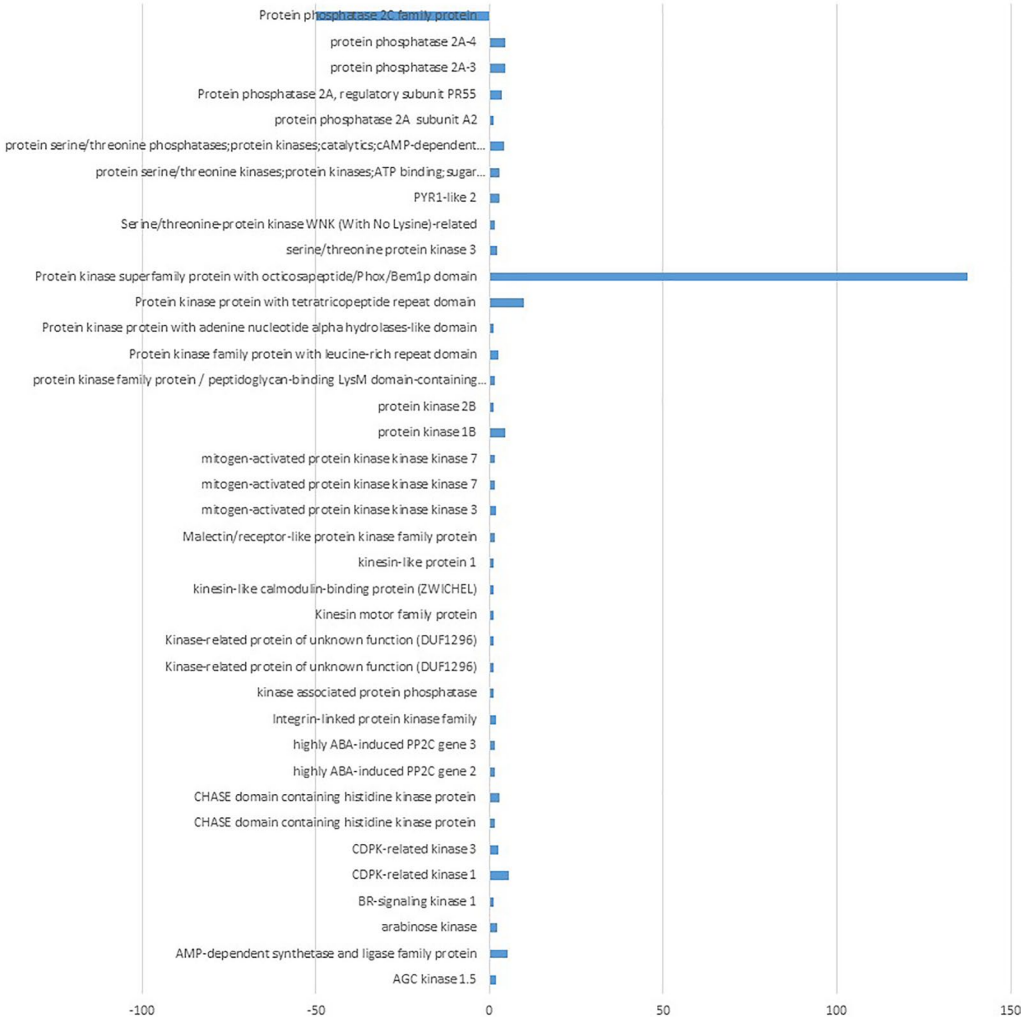

Fig. 5 Transcription level of genes encoding proteins related with kinases and phosphatases. The values represent the means of three replicates and are with respect to control level = 0%. Only the significant values at $p < 0.05$ by *t* test are shown

and phosphatases activity, and functional and regulatory (TFs) stress-responsive genes (Figs. 4, 5, 6 and 7).

It was observed that seedlings treated with HA showed high transcription level of genes related to stress perception, including free Ca^{2+} (Fig. 4), and different phytohormones, besides ABA, including auxins, ethylene, brassinosteroids and gibberellins (Fig. 4). In a previous electrophysiology study, we detected the free cytosol Ca^{2+} pulse in rice seedlings treated with HA from vermicompost [55], as well as a larger CDPK activity and transcription level of plasma membrane Ca^{2+} transporters. Genes related with these processes were induced by HA on maize seedlings in the PS (Figs. 4 and 5). In response to various environmental stimuli, the cytosolic Ca^{2+} concentration in the plant increased rapidly [75, 76], and it was sensed by several Ca^{2+} sensors, including calmodulins (CaMs) and other calcium-binding proteins (CaBPs). In plants, the calcineurin B-like protein (CBL) family represents a unique group of calcium sensors and plays a key role in decoding calcium transients, by specifically interacting with and regulating a family of protein kinases (CIPKs) [77, 78]. Calmodulin (CaM) is also involved in the transduction of Ca^{2+} signals and it is related to stress responses playing a central role in adaptation to adverse environmental conditions, including modulation of TF, such as WRKY (transcription factor involved in a key regulation of processes related to abiotic stress response) and several kinases and phosphatases activities, which act as an integrator of different stress signaling pathways [76]. Caleosin-related protein family was observed in high transcription level in PS of maize seedlings, while its involvement was previously observed in the negative regulation of ABA responses in Arabidopsis [76].

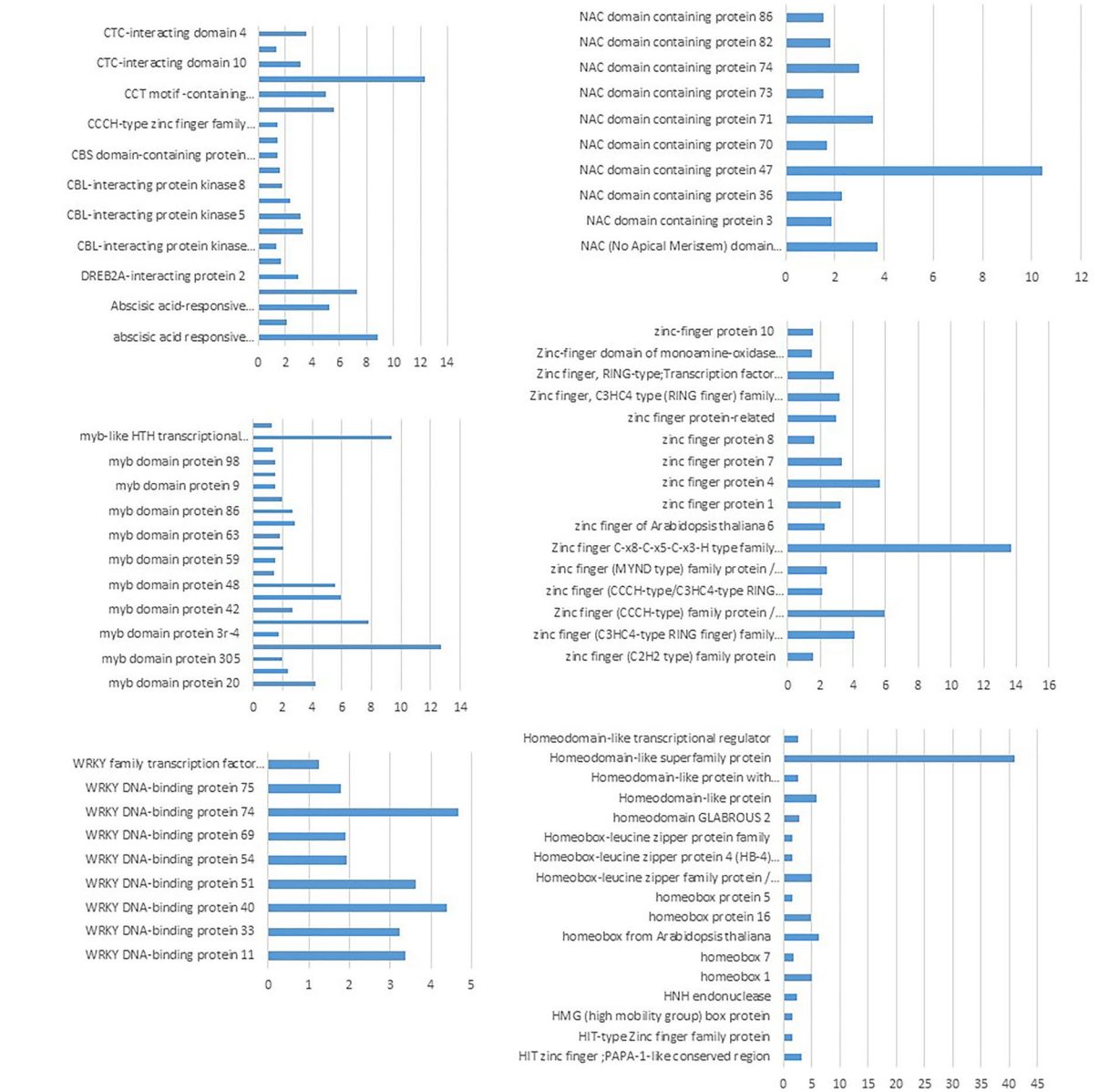

Fig. 6 Transcription level of genes encoding proteins related with transcription factors ABA-dependent and independent. The values represent the means of three replicates and are with respect to control level = 0%. Only the significant values at $p < 0.05$ by *t* test are shown

Abiotic stress responses are largely regulated by the five well-known plant hormones: auxin, ethylene, cytokinin, abscisic acid and gibberellins [78]. A high transcriptional level of Small Auxin-Up RNA (SAUR)-like gene (Fig. 4) was observed. This was a typical response observed in Arabidopsis treated with ACC (a precursor of ethylene) [79], thus indicating a putative ethylene/auxin crosstalk mechanism induced by HA. The relevance of primary control occurs by activation of genes that contain auxin- and ethylene-responsive elements to HA, as shown in Fig. 4. The pivotal role of ethylene on plant growth and abiotic stress response was discussed by Dubois and colleagues [80], who showed that an increasing number of transcriptome studies in plants exposed to abiotic stress suggested a role for ethylene under a broad range of stresses. Plant steroidal hormones like brassinosteroids were also involved in the plant abiotic stress response, including molecular mechanisms that confer tolerance against heat, cold, drought, and salt stress [81]. We also observed the

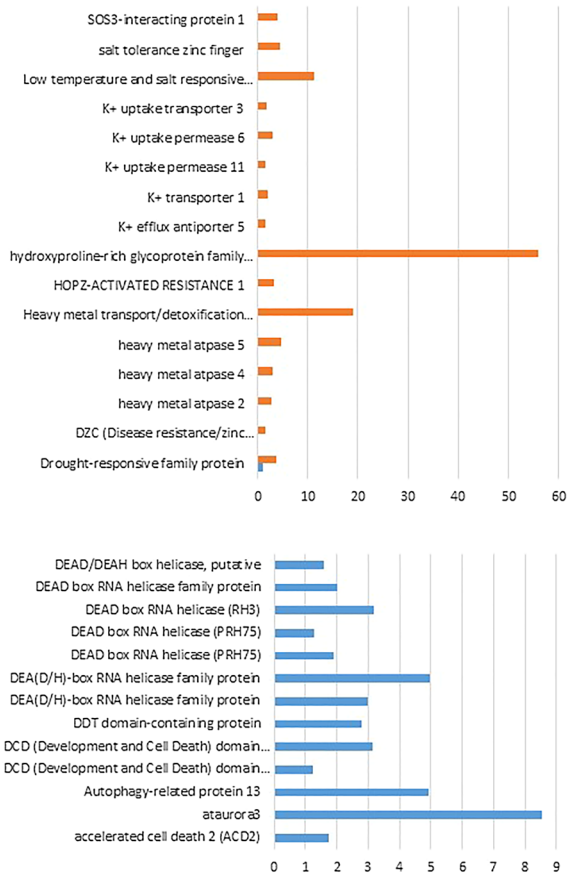

Fig. 7 Transcription level of genes encoding proteins related with functional stress-responsive genes and autophagy process. The values represent the means of three replicates and are with respect to control level = 0%. Only the significant values at $p < 0.05$ by *t* test are shown

transcriptional level of Brassinosteroids element binding that was enhanced by HA treatment (Fig. 5).

However, the most popular hormone involved in anti-stress responses is ABA that is known as the stress hormone. In fact, we found the ABA element binding in high transcriptional level in PS (Fig. 5). We also observed the presence of ABA-dependent mechanism of stress response since the PYR1-like 2 and ABA receptor were induced in the PS (Fig. 4) The transcriptional level of PYR1-like 2 was twofold larger than control. According to Zelicourt et al. [82], the primarily perception of hormone stimulus that activates downstream events is due to two protein classes, besides the ABA receptor per se, which are negative regulators of the protein phosphatase 2C (PP2C). This indicates that the ABA-induced inhibition of PP2Cs leads to SnRK2 autophosphorylation and activation of the positive regulators SNF1-related protein kinases type 2 (SnRK2s). Both the inhibition of PP2C and serine/threonine protein kinase stimulation was observed in our experiments (Fig. 6). While the enhancement of protein kinases transcription level induced by HA was previously observed [55], we can now also include MAPKs (Fig. 5). In addition, under very restrictive nutritional conditions, the TOR kinase (target of rapamycin) expression was unusually induced by HA [83], thus indicating that cell growth and proliferation resulted in high shoot and root weight under low amino acids and sugars content. The role of TOR kinase on plant cell nutrition was elegantly described by Robaglia and collaborators [84]. They showed that TOR functions as a regulatory hub integrating environmental inputs, such as availability of nutrients, integrity of the cell and presence of proliferation stimuli that coordinate cell growth and proliferation.

After stress perception and cell signaling by kinases and phosphatases activities, the induction of stress-responsive gene expression in the PS of maize seedlings brought about by HA, through either ABA-dependent or ABA-independent pathways activated physiological and metabolic responses and further stress alleviation (Fig. 8). Generally, the stress-responsive genes can be classified in two types: (i) functional genes encoding important enzymes and metabolic proteins which directly protect cells from stresses, and (ii) regulatory genes encoding various regulatory proteins, including TFs which regulate signal transduction and gene expression in the stress response [85]. TFs are proteins that act together with other transcriptional regulators, including chromatin remodeling/modifying proteins, to employ or obstruct RNA polymerases to the DNA template [85]. The TFs interact with *cis*-elements in the promoter regions of several stress-related genes and thus upregulate the expression of many downstream genes, thus conferring an abiotic stress tolerance [85].

The TFs upregulated by HA revealed both ABA-dependent and ABA-independent pathways (Fig. 6). The expression of ABA-responsive genes is mainly regulated by bZIP TFs known as AREB/ABFs, MYC/MYB and WRKY, which act in an ABA-responsive element (ABRE) dependent manner and were found in high transcriptional level in PS maize seedlings (Fig. 6). MYB (myeloblastosis) family also participates in the ABA-dependent pathway involved in abiotic stress signaling for the control of stress-responsive genes. Kimotho and colleagues [86] provided strong evidences that these genes may take part in signal transduction pathways involved in abiotic stress responses in maize. The same authors reported that WRKY domain (largest superfamily of TFs only found in plants) shows a strong binding affinity for a *cis*-acting element known as W-box (TTGACC/T), which is present in a number of abiotic stress-responsive genes. The ABA-responsive element

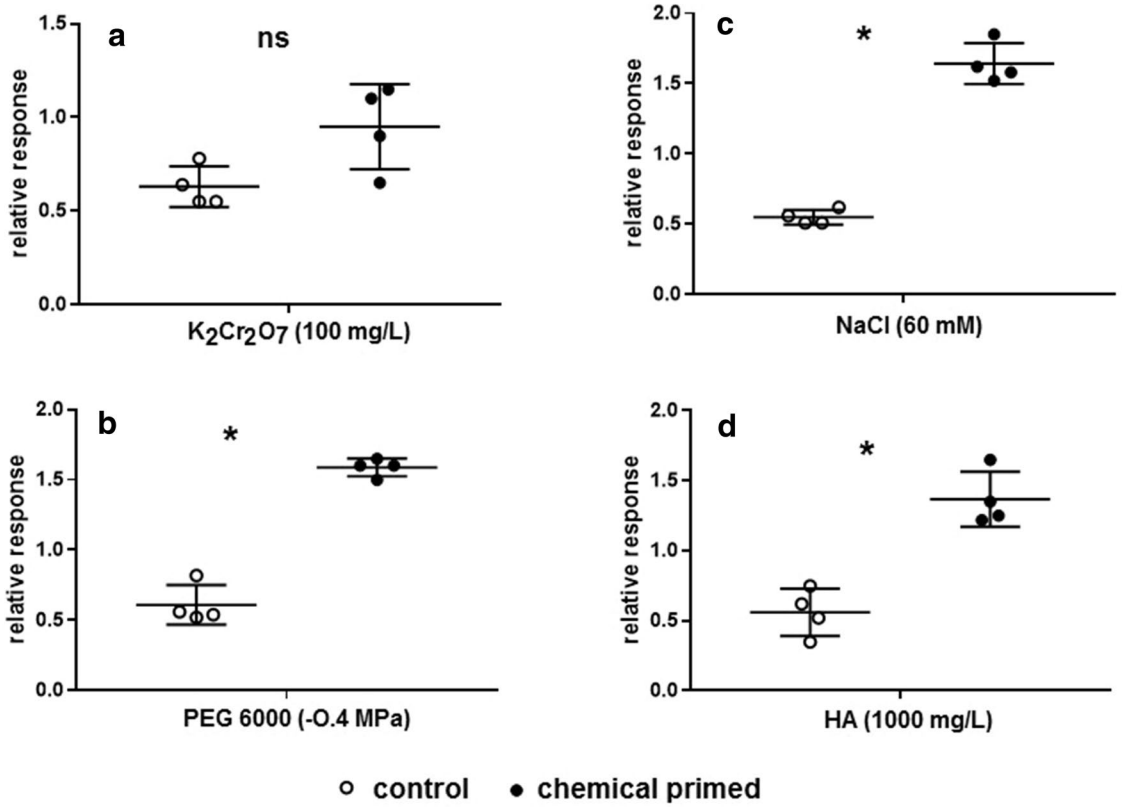

Fig. 8 Alleviation of root stress symptoms (fresh weight) due to plant preconditioning by humic acids against **a** chromium toxicity; **b** drought stress by PEG 6000; **c** salt stress by NaCl 60 mM and **d** high concentration of humic acids

(ABRE) is a conserved *cis*-acting element subjugated by the basic Leucine Zipper Domain (bZIP) TFs. bZIP TFs, which are part of the AREB/ABF regulons. They give an excellent example of interactions involving stress-responsive genes which carry the *cis*-acting element (ABRE) whose exogenous expression led to significant tolerance to freezing, salt, oxidative stress and drought in Arabidopsis transgenic plants [6].

The ABA-independent TFs (DREB, CBF, NAC and ZF) were also upregulated in primed maize by HA (Fig. 5). The roles of NAC TFs in plants have been extensively studied in rice and *Arabidopsis*. In maize, the *ZmSNAC1* gene was strongly induced by high salinity, drought, and ABA [87]. Over-expression of *ZmSNAC1* in transgenic *Arabidopsis* led to increased hypersensitivity to osmotic stress and ABA. An enhanced tolerance to dehydration stress suggests that NAC TFs are a multiple stress-responsive actor that positively modulates abiotic stress tolerance in maize [88]. The dehydration-responsive element binding proteins (DREBs) play a significant role in the ABA-independent pathways. They also take part in the induction of abiotic stress-associated genes, thus resulting in abiotic stress tolerant plants [88].

HD-Zip proteins represent a large TF family that is specific to plants. The expression of Zmhdz10 (the first HD-Zip isolated from maize) was activated by ABA and enhanced salt and drought tolerance [89]. This is in line with the high proline concentration that was observed in the priming phase of this experiment (Fig. 3). The ultimate consequences of TFs activation induced by HA were (i) the expression of stress-responsive genes without the presence of abiotic stress and (ii) high transcriptional level of genes encoding for autophagy. In this regard, we found here the gene stress-responsive expression in PS seedlings induced by HA without the presence of any stressor agent (Fig. 7). We observed a high level of SOS3 (Fig. 7) gene that encodes a myristoylated calcium-binding protein responsible for sensing cytosolic calcium changes that are elicited typically by salt stress [90]. In addition, SOS3 physically interacts with and activates SOS2 requiring calcium [90], being consistent with the role of calcium as second messenger in stress responses and with the high level of calcium-sensing proteins induced by HA (Fig. 4). In addition, it was observed that HA induced a TF involved in a salt stress response, like salt tolerance zinc finger (Fig. 7).

As the specific functional and regulatory gene response against salinity were induced by HA, it was expected that the cell transporters should be also affected, since the osmotic stress is a first consequence of cell ion imbalance. In fact, unspecific K^+ uptake transporter, K^+ transporter, K^+ antiporter, K^+ permease and K^+ transporter were all transcripted at high level with respect to control (Fig. 7). The Na^{+}/K^+ antiport is activated for salt cell detoxification while the high-affinity K^+ transporter 1 (HAKT1) was induced by HA in Arabidopsis and was involved in a salinity tolerance mechanism [25]. Moreover, the extensions hydroxyproline-rich repetitive glycoproteins are essential to root elongation [91] and alleviation of turgor pressure due osmotic imbalance. The high level of transcription was also observed in PS induced by HA (Fig. 7).

ATPases induced by heavy metals (HMA) were found to be in high level (Fig. 7), including heavy metal transport/detoxification family in the PS. These transporters have an important role in the heavy metal detoxification [92]. Two genes related to disease response were found in high transcriptional level in PS, such as HOPZ-activated resistance and DZC (disease resistance, zinc finger). The first one was considered key to the surveillance system against plant pathogens [93, 94], while the second one is also a classical resistance (R) gene type of defense and previously involved in defense against necrotrophic fungal pathogens including *Pseudomonas* [93]. In common both of these disease gene responses are linked to leucine-rich (LR) TFs which are highly induced by HA.

Finally, we found a group of genes encoding proteins related to autophagy induced by HA. DEA (D/H) box RNA helicase family protein, DEAD/DEAH box family, autophagy related protein 13 (ATG), Atauor a3, accelerated cell death (ACD), and DCD (development and cell death) were found in high transcription level (Fig. 7). According to Linder's review [95], DEAD-box proteins play important roles in RNA metabolism, including the transcription to the degradation of RNA, and pre-mRNA splicing, mRNA export, ribosome biogenesis, translation initiation and gene expression in organelles Finally, some DEAD-box proteins may function as a sort of 'check point' control for the correct functionality to avoid erroneous splicing or protein synthesis.

ACD genes were linked to reduction of diseases symptoms and alleviation of cell damage induced by ROS [96]. Another plant gene response to infection requires salicylic acid as the signaling compound downstream of the recognition process to proceed beyond restriction points in the cell death program activated by DCD complex [97]. It is well known that HA can induce the PAL/TAL expression, thus enhancing the phenolic content in plants [63], including salicylic acid concentration [13] (Additional file 3: S3). The core of autophagy management to crop protection resides in the plant response against abiotic and biotic stress that includes the local activation of response system to prepare plants cells for the next stress [98]. The latter authors reviewed the autophagy process in relation with its critical role in the development and stress responses, showing that manipulation of autophagy in crop plants may eventually lead to beneficial agricultural applications. They highlighted the pivotal role of ATG proteins in abiotic stress response, including drought and nutritional restriction. In addition, it was observed that decreasing the expression of target of rapamycin (TOR) is a negative regulator of autophagy and ATG-related genes [99]. This is in line with our previous observation of high expression of TOR in plants treated with HA, despite the nutritional regime (high/low) and low content of sugars and amino acids on plant tissues [83].

Conclusion

The use of biostimulants in agriculture has grown steadily from the last decade around 10% or more a year, whatever the indicator used (sales, treated hectares, number of users). Together with plant growth stimulation, crop protection against abiotic stress is reported as one of the main plant effects. Humic acid-biostimulant formulations are strongly dependent on concentration rate and plant species, which ultimately can modulate plant defense mechanisms and are widely used to alleviate the effects on plants of salinity, drought and heavy metals toxicity (see Table 1). We postulated that HA can be used as a chemical priming plant defense agent. Maize seedlings treated with HA showed typical hormesis response, based on biochemical markers at preconditioned experimental phase with biphasic dose–response. We found a fit between the best dose–response for root growth promotion (fresh weigh increase), and proline accumulation and catalase activity. The transcriptomic analysis of PS induced by HA showed a significant transcription level of genes encoding stress perception and cell signalization, including kinases, phosphatases proteins, and functional and regulatory (transcription factors) proteins, which are involved in gene response against abiotic stress, including those linked to the autophagy process. The further exposition of chemically primed maize seedling to abiotic stress agents resulted in a clear increase of plant tolerance, also at large HA concentration (Fig. 8). The hormesis action of HA extract in maize seedlings is summarized in Fig. 9. This work provided an experimental evidence that helps understanding the chemical priming effect by HA in maize seedlings. This implies a potential future research direction to apply this concept to crop stress management.

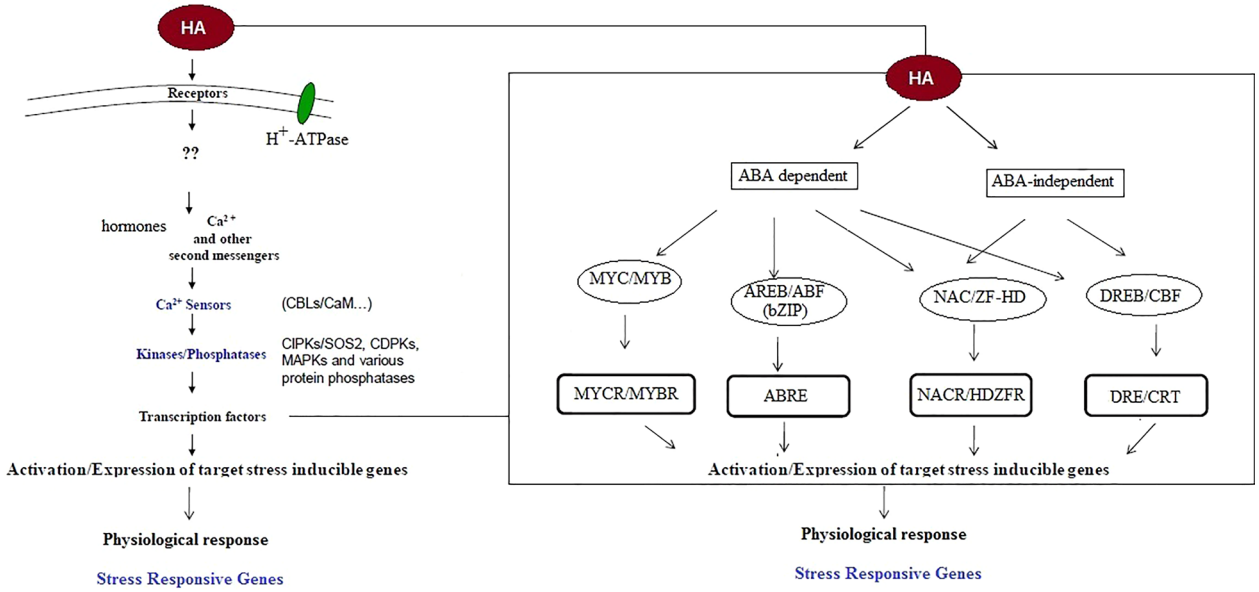

Fig. 9 Schematic representation of primed state (PS) induced by humic acids (HA). Cell receptors are sensitized triggering cellular messengers associated with stress signal amplification (Ca^{2+} signaling and hormonal pathway) triggering a cascade of kinases and phosphatases-induced signaling resulting in a high level of transcription of regulatory (transcription factors) and functional stress response genes

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40538-020-00178-4.

Additional file 1. CP/MAS ^{13}C NMR spectrum of humic acids isolated from vermicompost.

Additional file 2. Stress alleviation symptoms in shoot maize seedlings preconditioned by humic acids.

Additional file 3. Phenylpropanoids pathways in maize seedlings primed by humic acids.

Abbreviations

CP/MAS ^{13}C NMR: Cross-polarization/magic-angle spinning nuclear magnetic resonance of isotope carbon with 13 mass; HA: Humic acids isolated from vermicompost; ROS: Reactive oxygen species.

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Authors' contributions

LPC, FLO and AP were responsible by experimental idea. NOAC carried out the experiments and conducted the biochemical analysis; LESSI was responsible for transcriptomic analysis. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

This manuscript is an original paper and has not been published in other journals. The authors agreed to keep 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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