

REVIEW

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Humic substances and plant abiotic stress adaptation

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Abstract

Background Traditional agriculture is on the front line of climate change, being most impacted by the increase in the intensity and frequency of extreme events, such as floods, drought and rising temperatures. Local ecological knowledge is a recognized keystone of successfully managed socioecological systems, but loss of soil fertility, water scarcity, incidence of diseases and decreased production due to climate change are linked to the greater vulnerability experienced by traditional farmers. Plant biostimulants are natural products used to stimulate nutrient uptake and efficiency by crops, increase tolerance to abiotic/biotic stress and improve quality without negative impacts on the environment if obtained from renewed sources. Humic substances are some of the most used plant biostimulants in agriculture and play a central role in plant adaptation.

Materials and methods We reviewed and discussed a sample set of papers ($n = 52$) about humic substances to mitigate abiotic stress in crops using data basis from Web of Science (Clarivate Analytics), Scopus—IBM (International Business Machines Corporation), and Scielo (Scientific Electronic Library Online).

Results The predominance of authors in the global south is notable, but it is not a coincidence, since this is where the effects of climate change will have the greatest impact. The mechanisms involved in the stress mitigation involve the activation of signaling factors, gene response induction, the accumulation of osmoprotective and anti-oxidant compounds, the induction of antioxidative metabolism, ion homeostasis, membrane transport and adjustment of hormonal balance. The intriguing question is: how can a complex mixture of molecules affect so many distinct effects on plants responsible for plant adaptation?

Conclusions The complexity of humic substances challenges our knowledge method, but supramolecular chemistry may provide answers that enable us to broaden our understanding of the plant defense mechanisms modulated by these substances.

Keywords Climate change, Small-scale producers, Plant biostimulants, Physiological effect, Humic acids

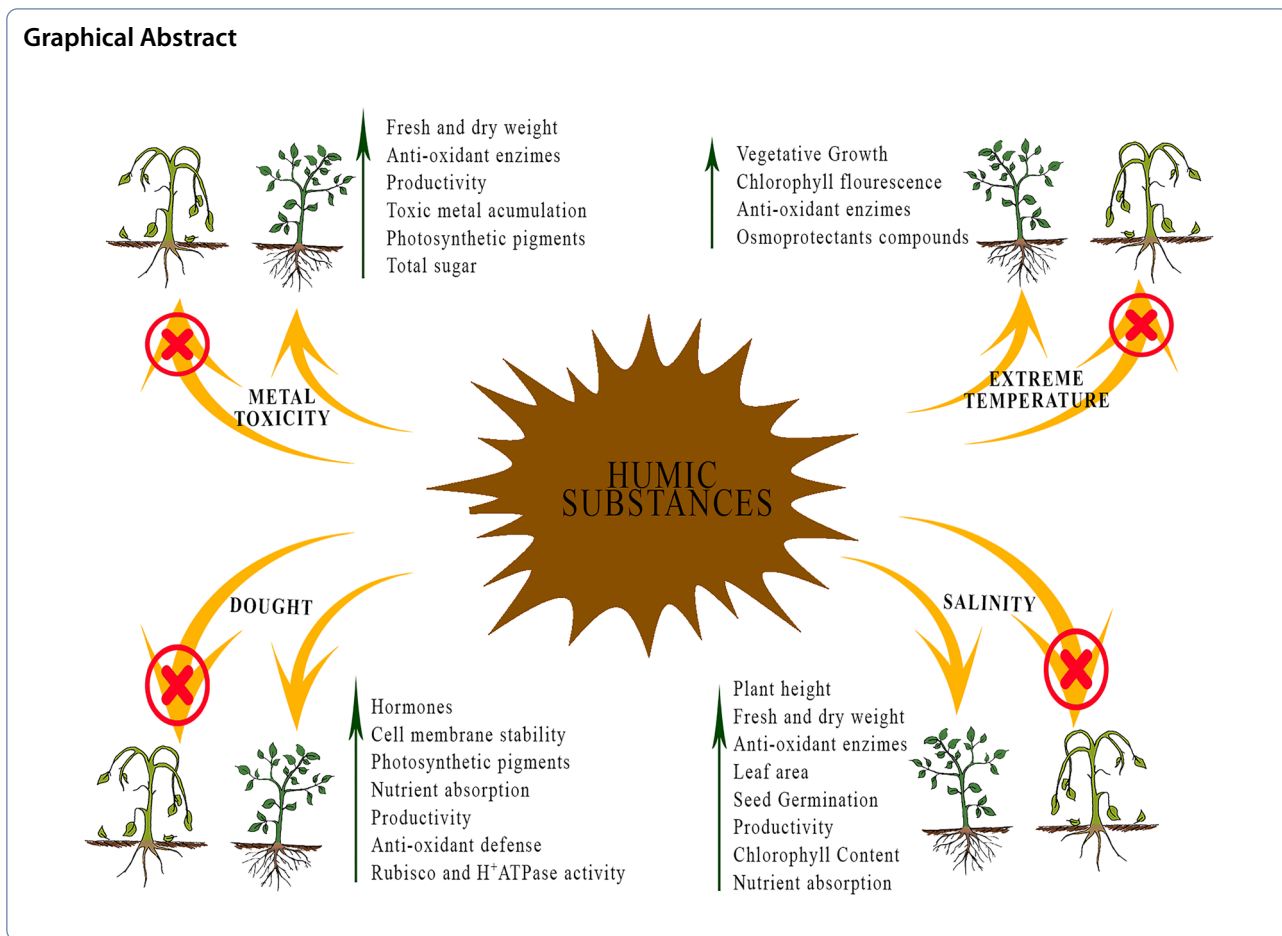
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Introduction

Small-scale producers represent 80% of global rural properties and produce more than 80% of the food consumed in developing countries [1]. They are on the front line of climate change [2], being most impacted by the reduction in crop productivity and other damages resulting from the increase in intensity and frequency of extreme events, such as floods, droughts, and frosts [3]. Extreme weather conditions have shaken the lives of small rural communities, a trend attributed to climate change and human actions (<https://news.un.org/pt/story/2021/08/1760132>). For example, high temperatures and lack of rainfall in the Alto Rio Negro region, in the west of Amazonas, have changed the centuries-old practices of working in the fields, putting the food security of entire communities at risk (<https://www.bbc.com/portuguese/articles/c6pjn19jw9xo>). The intensity and frequency of extratropical cyclones have caused floods with devastating consequences for agricultural production in southern Brazil (<https://www.bbc.com/portuguese/articles/c2qlypx3k1wo>). The planet is getting warmer, consequently so are the ocean waters,

and El Niño is evolving. This combination is causing the atmospheric conditions that make meteorological events more intense.

Traditional family-based agriculture is more vulnerable to climate extremes. This, in turn, leads to greater poverty levels, food inflation and unemployment, posing serious risks to food and nutritional security, especially where agriculture is the most relevant economic activity [3]. In Brazil, 33 million people (15% of the population) is considered to be in serious food and nutritional insecurity in 2022, the majority of them living in rural areas [4]. The vulnerability is even more dramatic as traditional knowledge based on experience and on the observation of nature has been put to the test by ongoing climate change. Adapting traditional crops to unexpected impacts is an important achievement.

The frequency and severity of climate events increased plant performance constraints, reducing crop productivity and quality [5]. Nowadays, abiotic stresses in plants are the leading cause of severe yield losses ranging between 50 and 80%, depending on the crop and geographical location [6]. The intensification

of agricultural production is expected to increase productivity in affected areas [7]. However, agricultural intensification is among the main drivers of climate change and soil contamination, making food production both the culprit and the victim [8]. Innovative technologies are essential to reduce agricultural pressure on the environment by decreasing water use and chemical inputs, while maintaining productivity [9]. The debate around sustainable food production should include appropriate goals to favor an agroecological transition. Biological inputs play a prominent role in this context since they promote plant growth, improve crop productivity and potentially reduce damages from climate change, allowing reduced employment of agrochemicals and of products from non-renewable origins.

Plant biostimulants (PB) can be considered an environmentally friendly agronomic tool to enhance abiotic stress tolerance, address environmental concerns, and fulfil the need to develop sustainable agriculture [10]. Humic substances are one of the most used PB. They are part of a market that grows 10% per year, which is expected to make US\$ 4.14 billion by 2025 (<https://www.grandviewresearch.com/press-release/global-biostimulants-market>). Traditional knowledge about organic matter management allowed us to evolve from gatherers and hunters to drivers of social development [11]. In this regard, the use of humic substances (HS), a fraction of soil organic matter and organic residues, can enhance production, and this effect is significantly higher under stress conditions regardless of the sources of extraction [12, 13]. Humic products (HP) from renewable sources (e.g., raw materials from agro-industrial processing or animal husbandry) are highly bioactive and can be used to substitute commercial products based on coal, lignite or peat [14, 15]. Aspects related to the use of HPs to mitigate the effects of stress on plants will be addressed in this review.

Abiotic stress and humic substances

Under natural conditions, plants usually face different environmental stresses, such as drought, salinity, extreme temperatures, heavy metals, and UV radiation [5]. Previous studies have demonstrated the promising potential of HPs, as well as their functions and possible challenges to mitigate different abiotic stresses and improve quality and yield [10, 16, 17]. Table 1 summarizes the scientific reports found using the terms “*humic*”, “*plant*”, “*heavy metals*”, “*salinity*”, “*drought*”, “*extreme temperature=heat/cold*”, and “*UV radiation*”, published in the last 6 years (2018–2023). For this purpose, three data bases (Web of Science, Google Scholar and Scopus)

were employed. The purpose of this bibliographic review was not to thoroughly explore the available databases but rather to provide a few examples on how HP can contribute to reduce abiotic stress damages.

General damage promoted by abiotic stress and general plant response

In general terms, all forms of biotic and abiotic stress promote the generation of reactive oxygen species (ROS). The production of ROS in cells under normal growth conditions is low ($240 \mu\text{M s}^{-1} \text{O}_2^-$ and a steady-state level of $0.5 \mu\text{M H}_2\text{O}_2$ in chloroplasts), but different stresses including drought, salinity, high CO_2 concentration, heavy metals and nutrient deficiency can disrupt the cellular homeostasis, enhancing the production of ROS ($240\text{--}720 \mu\text{M s}^{-1} \text{O}_2^-$ and a steady-state level of $5\text{--}15 \mu\text{M H}_2\text{O}_2$) [63]. The cellular response against the resulting oxidative stress is critical for plant growth and adaptation [64]. Oxidative stress generally occurs when the balance between the production of ROS and the quenching activity is upset by a stressful event. If the stress level reaches a threshold, the excessive accumulation of ROS will trigger gene response, including programmed cell suicide pathways [65]. Stress-induced ROS production is part of a multilayered reduction/oxidation (redox) response system in which stress sensing and adaptation are synchronized with plant metabolism and phytohormone pathways [64]. Moreover, any physical, chemical, or metabolic shock activates the plasma-membrane-bound NADPH oxidases and apoplastic peroxidases, leading to an oxidative burst. A complex anti-oxidant network has evolved in plant cells to scavenge ROS and regulate their levels according to the requirements of cell signaling. The main mechanism of stress tolerance modulated direct or indirectly by HS are shown in Box 1. Certain aspects of the following topics are discussed.

Box 1. Some of the general abiotic stress tolerance mechanisms induced by humic substances

- Activation of signaling factors;
 - Gene response induction;
 - Accumulation of osmoprotective and anti-oxidant compounds;
 - Induction of antioxidative metabolism;
 - Ion homeostasis and membrane transport;
 - Adjustment of hormonal balance
-

Activation of signaling factors under stress: three masters cell messages are influenced by humic substances

Stress perception involves cell receptor activation, and the amplification of signal recruits second messengers, so-called because they represent intracellular signals being translated from the primary external signal.

Table 1 Scientific reports published in the last 6 years (2018–2023) considering the attenuation effect of humic substances against different abiotic stress damage (drought, salinity, extreme temperature, UV-radiation and heavy metal toxicity)

Plant	Assay	Source	Concentration range	Application	Summary of main effects	Reference
Drought						
Fava beans (<i>Vicia faba</i> L.)	F	CP	10 kg ha ⁻¹	Soil	Increased cell membrane stability, photosynthetic pigments, induction of osmoprotective activity (proline and catalase) and nutrient absorption	[18]
Wheat (<i>Triticum aestivum</i> L.)	F	CP	60 kg ha ⁻¹	Soil	Increase in grain productivity by approximately threefold	[19]
Beet (<i>Beta vulgaris</i>)	F	CP	30 L ha ⁻¹	Soil	Increased root length, root and shoot fresh mass, larger leaf area and sugar production per hectare	[20]
Soybean (<i>Glycine max</i> L.)	G	HA	5 mg HA dm ⁻³	Nutrient solution	It triggered the anti-oxidant defense system and increased morphological characteristics, such as the plant's fresh and dry matter of shoots and roots, as well as root length and height	[21]
Maize (<i>Zea mays</i> L.)	G	HA	45 kg ha ⁻¹	Soil	Increased productivity, photosynthetic parameters, Rubisco and ATP synthase activity, water use efficiency, nutrient availability in soil, soluble sugars, trehalose, proline, betaine, IAA and ABA	[22]
Durum wheat (<i>Triticum durum</i> L.)	F	CP	NI	Foliar spray	5% increase in grain yield under water stress	[23]
Onion (<i>Allium cepa</i> var. Zargan)	G	HA	100 mg kg ⁻¹	Soil	Increased concentration of soluble sugars and higher activity of antioxidant enzymes (peroxidase, catalase and superoxide dismutase)	[24]
Potato (<i>Solanum tuberosum</i>)	G	CP	4.5 L ha ⁻¹	Soil	Increased production of fresh tubers by 63.48% compared to control and improved photosynthesis parameters	[25]
Rice (<i>Oryza sativa</i> L.)	F	CP	10 kg ha ⁻¹	Foliar spray	Reduced oxidative damage caused by ROS, protecting the physiological processes of plants	[26]
Millet (<i>Pennisetum glaucum</i>)	F	HA	4.5 L ha ⁻¹	Foliar spray	Larger millet yield and reduced root-shoot ratio	[27]
Maize (<i>Zea mays</i> L.)	Lab	HS	1% (v/v)	Foliar spray	Promoting anti-oxidant capacity and proline levels in plants, thus improving tolerance to water stress	[29]
Pepper (<i>Capsicum annuum</i>)	F	HA	4.5 L ha ⁻¹	Soil	Higher accumulation of aboveground part biomass of pepper, tomato and lettuce and acceleration of growth rates	[28]
Melon (<i>Cucumis melo</i> L.)	F	HA	300 mg L ⁻¹	Substrate	Promotion of the anti-oxidant activity of enzymes superoxide dismutase, catalase and glutathione reductase	[30]
Purple flower (<i>Echinacea purpurea</i> L.)	G	HA	500 mg L ⁻¹	Substrate	Increased antioxidant activity, total flavonoid content, chlorophyll a, and carotenoid content	[31]

Table 1 (continued)

Plant	Assay	Source	Concentration range	Application	Summary of main effects	Reference
Salinity						
Quinoa (<i>Chenopodium quinoa</i> L.)	F	HA	1% (v/v)	Substrate	Compared to the control, more significant plant height, dry matter, and fresh weight of quinoa plants	[32]
Rice (<i>Oryza sativa</i> L.)	G	HA	100 mg L ⁻¹	Seed conditioning + soil	It increased the production of ROS and the activity of antioxidant enzymes (peroxidase and catalase), and root growth	[33]
Cotton (<i>Gossypium hirsutum</i> L.)	G	FA	450 kg ha ⁻¹	Substrate	Improved leaf area index, stem diameter and plant height; also increased cotton seed yield by 6.22%	[34]
Papaya (<i>Carica papaya</i> L.)	G	HA	3.5 mL L ⁻¹	Substrate	It attenuated the deleterious effects of saline stress, promoting growth and improving the performance of papaya seedlings under moderate salinity (4 dS m ⁻¹)	[35]
Rice (<i>Oryza sativa</i> L.)	G	HA	40 mg L ⁻¹	Foliar spray	Reduction of Na ⁺ toxicity by increasing the K ⁺ /Na ⁺ ratio, regulating the concentration of osmolytes and increasing the activities of antioxidant enzymes	[36]
Maize (<i>Zea mays</i> L.)	G	HA	50 mg L ⁻¹	Seeds	Seed germination uniformity and maize seedling growth under saline conditions	[37]
Wheat (<i>Triticum aestivum</i> L.)	G	HA	200 mg kg ⁻¹	Substrate	Increase in wheat productivity	[38]
Papaya (<i>Carica papaya</i> L.)	G	HS	20 g kg ⁻¹	Substrate	Increased CO ₂ concentration, transpiration rate, instantaneous water use efficiency, carboxylation efficiency and chlorophyll b content	[39]
<i>Terminalia arjuna</i>	F	HA	NI	Substrate	Increase of fresh biomass and photosynthetic parameters, such as chlorophyll a and b and carotenoids	[40]
Maize (<i>Zea mays</i>)	F	HA	3.75 ton ha ⁻¹	Substrate	Increased salt tolerance of corn roots, greater availability of soil nutrients and increased grain yield	[41]
Garlic (<i>Allium cepa</i> L.)	G	HA	1.0 g kg ⁻¹	Substrate	Increased the contents of some nutrients (K, Ca, N, P, Mg, Fe, Zn and B), reduced Na ⁺ toxicity and increased plant growth	[42]
Melon (<i>Cucumis melo</i> L.)	F	HA	21 L ha ⁻¹	Substrate	More significant length, fresh and dry mass, chlorophyll (SPAD), fruit mass and increase in the content of foliar nutrients (N and K) and total soluble solids (TSS) on fruits while reducing the Na ⁺ content of the leaves	[43]
Extreme temperature						
<i>Arabidopsis thaliana</i>	Lab	CP	860 mg L ⁻¹	Substrate	Induction of heat stress tolerance gene expression, such as heat shock protein	[44]

Table 1 (continued)

Plant	Assay	Source	Concentration range	Application	Summary of main effects	Reference
Tomato (<i>Solanum lycopersicum</i>)	Lab	HA	500 mg L ⁻¹	Substrate	Increased vegetative growth and chlorophyll fluorescence, anti-oxidant enzymes (APX, SOD, GSH and LPO) and the expression of genes, such as SIWRKY33b, SIHKT1 and SIATG5 related to thermal tolerance	[45]
Coriander (<i>Coriandrum sativum</i> L.)	G	HA	50 mg L ⁻¹	Substrate	growth promotion and increase of anti-oxidant and osmoprotectants compounds, such as sugars, carotenoids, flavonoids, phenols and polyphenols, minimizing the effect on plants	[46]
Heavy metal toxicity						
Wheat (<i>Triticum aestivum</i>)	F	HA	40 mg Kg ⁻¹	Substrate	Increase of 65.64 and 71.48% of fresh weight and dry weight in wheat, respectively, compared to the control where the plants were under Cd stress and did not receive HA treatment	[41]
Water plant (<i>Vallisneria natans</i>)	Lab	HA	2 mg L ⁻¹	Substrate	Increased growth effectively induced anti-oxidant responses and SOD, POX and MDA protein synthesis and alleviated toxicity by complexation with Cd and Pb metals	[47]
Maize (<i>Zea mays</i>)	F	CP	NI	Substrate	Increase of 44.20% in productivity compared to the control; decrease of Cd accumulation by 20.19%	[45]
Rice (<i>Oryza sativa</i> L.)	G	CP	1% (p/p)	Substrate	Increase the activity of antioxidant enzymes, reduce Cd absorption and toxicity, and promote growth	[48]
<i>Dimorphotheca ecklonis</i>	G	HA	200–400 mg L ⁻¹	Substrate	Increase of growth parameters, photosynthetic pigments and total sugar content	[49]
Strawberry (<i>Fragaria</i> spp.)	G	HA	5 mM	Substrate	Reduction of membrane permeability, leaf temperature, proline levels and lipid peroxidation, attenuating the effect of Cd toxicity	[50]
Rice (<i>Oryza sativa</i> L.)	G	HS	200 mg C kg ⁻¹	Substrate	Translocation of MeHg in rice tissues by 88.95%, decrease in grain accumulation by 28.43%	[51]
Rice (<i>Oryza sativa</i> L.)	G	CP	1–4 g C kg ⁻¹	Substrate	Decrease of Cd availability and in translocation factor, decreasing the accumulation of Cd in the grain	[52]
Rapeseed (<i>Brassica napus</i> L.)	G	HA	2000 mg kg ⁻¹	Substrate	Increase in growth parameters, reduction in the content of heavy metals in roots and shoots, Reduction of CAT and APX enzyme activities and H ₂ O ₂ content in rapeseed	[53]
Garden cress (<i>Lepidium sativum</i>)	G	HS	7000 mg L ⁻¹	Substrate	An increased fresh and dry weight of leaves and roots stem diameter and leaf area; reduced Cd absorption by 95% and Cl absorption by 80%	[54]

Table 1 (continued)

Plant	Assay	Source	Concentration range	Application	Summary of main effects	Reference
Tea (<i>Camellia sinensis</i> L.)	Lab	HA	13.65 mg L ⁻¹	Nutrient solution	Regulation of cell wall synthesis and strengthening of the anti-oxidant system	[55]
Wheat (<i>Triticum aestivum</i>)	Lab	HS	750 mg L ⁻¹	Nutrient solution	Promotion of enzymes and non-enzymatic substances, including ascorbate–glutathione cycle, such as APX, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione, in addition to increasing GSH/GSSG indices	[56]
Wheat (<i>Triticum aestivum</i>)	Lab	HA	750 mg L ⁻¹	Nutrient solution	Promotion of SOD, CAT and NADPH-NOX oxidase enzymes and the ascorbate, glutathione and GSH/GSSG ratio	[57]
Mastic (<i>Schinus terebinthifolius</i>)	Lab	HS	8.57 mM C L ⁻¹	Substrate	Attenuation of iron toxicity. Development of root architecture, improving nutrient absorption and less iron accumulation in the biomass	[58]
Triticale (<i>Triticosecale wittm</i>)	Lab	HÁ	500 mg L ⁻¹	Nutrient solution	Reduction in the concentration of free amino acids; increase in conjugated and bound polyamine fractions; consequent reduction of oxidative stress caused by UV	[59]
Pepper, tomato, watermelon, and lettuce	F	HS	1% (v/v)	Substrate	Increased total biomass after transplanting, lower root/shoot ratio and higher root length and surface area	[28]
<i>Arabidopsis thaliana</i>	Lab	CP	860 mg L ⁻¹	Substrate	Induction of expression of heat stress tolerance genes, such as HSP	[44]
Tomato (<i>Solanum lycopersicum</i>)	Lab	HA	500 mg L ⁻¹	Substrate	Increased vegetative growth and chlorophyll fluorescence, anti-oxidant enzymes (APX, SOD, GSH and LPO) and the expression of genes, such as SIWRKY33b, SIHKT1 and SIATG5 related to thermal tolerance	[60]
Mungbean (<i>Vigna radiata</i>)	F	HA	60 kg ha ⁻¹	Substrate	It increased the activity of antioxidant enzymes, the expression of genes related to water and salt stress, and the efficiency of the photosynthetic apparatus	[61]
Maize (<i>Zea mays</i>)	Lab	HA	3.5–4 mM C L ⁻¹	Substrate	Higher root fresh weight, CTA activity promotion, and proline content enhancement. Promotion of gene response expression	[62]

The search was done on Scopus, Google Scholar and the Web of Science database

F field, G greenhouse, Lab laboratory, L leonardite, P peat, C compost, VC vermicompost, S soil, HS humic substances, HA humic acids, FA fulvic acids, CP commercial products

Other signaling components interpret these intracellular messengers further, resulting in the activation of downstream pathways that may have multiple outputs. The protein phosphorylation pathway activates

transcription factors (TF), inducing the expression of stress-responsive genes [66]. Several intracellular signaling molecules are involved in stress signal transduction. Reactive oxygen species, cytosolic Ca²⁺

concentrations, cell pH changes and phytohormones are among them. HS interact with many of these messengers, indicating their involvement in a regulation process that we do not entirely understand.

ROS play important signaling roles in the early stages of the stress response, activating stress-responsive genes that encode enzymes in anti-oxidant biosynthesis or enzymes that directly detoxify reactive oxidative radicals, mitigating stress damage [67]. Reactive oxygen species are demonstrated to regenerate Ca^{2+} signals to short and long distances by coordinating Ca^{2+} and ROS signals via Ca^{2+} -dependent phosphorylation of NADPH oxidases [68]. Upon exposure to various environmental stimuli, the cytosolic Ca^{2+} concentration $[(\text{Ca}^{2+})_{\text{cyt}}]$ increases rapidly, reaching micromolar levels; the transient influx of Ca^{2+} generates unique signatures that initiate cellular responses to diverse developmental cues and environmental challenges [69]. Because high $(\text{Ca}^{2+})_{\text{cyt}}$ is cytotoxic, $(\text{Ca}^{2+})_{\text{cyt}}$ is recovered within a range of 50–200 μM by Ca^{2+} -ATPases and $\text{H}^+/\text{Ca}^{2+}$ -antiporters [73] Lee and Seo. Then, Ca^{2+} is sequestered into vacuoles through an $\text{H}^+/\text{Ca}^{2+}$ antiport system driven by the proton-motive force of the tonoplast H^+ -translocating ATPase [70]. Felle [71] showed that pH also could act as a signal and/or a messenger of abiotic (changes in light intensity, drought, lack of oxygen) and biotic (presence of symbiotic partners or microbial attackers) factors. As a stress signal, pH involves transmembrane Ca^{2+} /pH interaction as a general principle of cellular signaling following the first encounter with defense-related substances. Perception of stress receptor activates G-protein, inducing Ca^{2+} influx, elevating cytosolic Ca^{2+} activity with the following consequences described by Felle [71]: (1) Ca^{2+} activates anion channels—a fraction of the anions that leave the cells and depolarize the plasma membrane are organic acids, which bind protons and thus alkalize the apoplast, leading to other transporters modulation; (2) elevated cytosolic Ca^{2+} activates an NADH oxidase, contributing to cytosolic acidification and external alkalization; and (3) elevated cytosolic Ca^{2+} and decreased pH are involved in gene activation.

The multiple messengers act simultaneously to activate stress signaling, and the most studied ones (ROS, Ca^{2+} and pH) can be influenced by the exogenous application of HS. Previous works have shown that HS can induce stress alleviation, promoting ROS accumulation and metabolism [16, 74, 75]. Applying HA extracted from vermicompost to rice seedlings increased the concentration of both H_2O_2 and O_2^- in specific root zones, accompanied by increased activity and gene expression of the main enzymes involved in ROS

metabolism [74]. Rice root seedlings exposed to humic acids (HA) showed a clear peak of Ca^{2+} influx in the same root zone of H^+ efflux coupled with very large anion exudation [76], unveiling evidence that HS influence $\text{H}^+/\text{Ca}^{2+}$ cell signaling. Phosphokinase Ca^{2+} dependent activity was monitored using differential gene expression, while voltage gate Ca^{2+} channels were also overexpressed in the presence of HP [76]. Moreover, Zandonadi et al. [77] showed that HA induce a concerted plasmalemma and tonoplast H^+ pumps activation in a typical control of cell electric environment mediated by phytohormones. Direct evidence of cytoplasmic pH changes induced by HP was obtained by Baia et al. [78] using a specific cytosolic pH (pH_{cyt}) dye. The pH_{cyt} was changed as a typical short chain organic acid stress triggered by HA.

The immediate consequence of stress signal transduction by secondary cell messengers is the activation of TF for stress gene response codification. The main TF related to abiotic stress response can be oversimplified into two categories: (1) Abscisic acid (ABA)-dependent including myeloblastosis oncogene (MYB) and myocytomatosis oncogene (MYC) regulon, ABA-responsive element binding protein (AREB) and ABA-binding factor (ABF) and (2) ABA-independent TF including NAC (NAM, ATAF1/2, and CUC2) family a plant-specific transcription factor involved in multiple abiotic-stress responses and zinc-finger homeodomain (ZFHD) regulon [79]. All these TF were induced in the maize seedlings treated with HA isolated from vermicompost [80]. Therefore, plants treated with HA showed high-stress response genes (drought, salinity, extreme temperature, heavy metals and pathogen response) transcription even without these stressors [62]. We do not know exactly how this occurs, but it is possible to offer certain explanations. Perhaps the most convincing one, because it is general and integrative, is the fact that HS can emulate the action of various plant hormones [81, 82], working as a key regulatory hub in plant responses integrating hormonal signaling and stress response pathways [80]. The following section provides examples of plant stress responses modified by HS.

Induction of low-molecular-weight anti-oxidant metabolites by humic substances

Plants accumulate large amounts of low-molecular-weight, anti-oxidant metabolites, such as ascorbate, glutathione, and tocopherol, and they have an extensive network of enzymatic anti-oxidants, such as superoxide dismutases, ascorbate peroxidases (APX), catalases, glutaredoxins (GRXs) and peroxiredoxins [83]. The evolution of this complex oxidant (ROS)/anti-oxidant network allows flexible control of cellular ROS levels. Tocopherol detoxifies ROS produced during oxidative

stresses and its biosynthesis take place through different pathways: the methylerythritol 4-phosphate, the shikimate (SK) and tocopherol-core pathways that are regulated by different enzymes [84]. The SK pathway is significantly modulated by HP, as demonstrated by Schiavon et al. [85]. Plants treated with HP showed high content of tocopherol and ascorbate [86, 87].

Lipid peroxidation is one of the most explored outcomes of ROS on membrane structure and function, and anti-oxidant metabolites protect the membrane by inhibiting lipids peroxidation [88]. Ascorbate (AsA) plays a vital role in stress physiology, especially in protecting lipids peroxidation [89]. It was observed that total AsA content was higher in plants treated with HA [57]. Ascorbate also maintains the membrane-bound anti-oxidant α -tocopherol in the reduced state [90]. The non-protein, water-soluble and low molecular weight tripeptide thiol glutathione (GSH; α -glutamyl cysteinyl glycine) plays a pivotal role in minimizing cellular dysfunction arising through stress-induced redox perturbation. Successive oxidation and reduction of Asa, glutathione and NADPH would enhance the potential scavenging of H_2O_2 generated through photooxidative stress in the chloroplast. These reactions are collectively called the ascorbate–glutathione cycle [91]. Increased activation of superoxide dismutase (SOD), catalase (CAT) and NADPH-oxidase (NOX) enzymes and ascorbate, glutathione (GSH) and GSH/GSSG ratio (The ratio of reduced GSH to oxidized GSH: GSSG) was observed in the presence of HA under Cd stress [57].

Promotion of compatible solutes biosynthesis

The availability of water for its biological roles as a solvent and a transport medium, an electron donor in the Hill reaction, and an evaporative coolant, is often impaired by environmental conditions. Although plant species display varying degrees of sensitivity to reduced soil water potential, low temperature or high salinity, it is assumed that all plants, at some level, have encoded capability for stress perception, signaling and response [92]. Osmoprotectants or compatible solutes are small molecules that act as osmolytes and help organisms survive extreme osmotic stress [93]. The main compatible solutes induced by stress include proline, citrulline, glycine betaine, 3-dimethylsulfoniopropionate, monosaccharide (fructose), sugar alcohols (mannitol and pinitol), and di- and oligo-saccharides (sucrose, trehalose and fructan) [94]. Metabolic acclimation via the accumulation of compatible solutes is often regarded as a primary strategy for the protection and survival of plants under abiotic stress [94]. Compatible solutes contribute to stress tolerance by acting as osmoregulators, since their high solubility in water substitutes for water molecules

released from leaves. In some cases, compatible solutes act as active oxygen scavengers or thermostabilizers [95]. The reports showing proline accumulation in plants treated with HS are abundant and mainly related to drought and salinity [96–105]. Additional changes that HS promotes on the carbohydrate profile include the production of non-reducing sugar trehalose [86], whose concentration increases under abiotic or biotic stress, acting in the cell osmoregulation [106]. Hassan et al. Aguiar et al. [103] also observed changes in carbohydrate profiles in sugarcane treated with HA after drought stress using a metabolomic approach. Three compounds linked to ascorbate metabolism–catabolism (vitamin C), threonic, isothreonic and oxalic acids, were also observed in greater concentrations in both maize and sugarcane leaves treated with HA in the presence of plant-growth-promoting bacteria [87]. The pathways by which ascorbate is catabolized to form oxalic, threonic and isothreonic acids have been previously reported, as well as their roles in many aspects of redox control and anti-oxidant activities in plant cells [104].

Activation of enzymatic anti-oxidant metabolism

Superoxide is scavenged via the disproportionation reaction catalyzed by SOD that produces hydrogen peroxide. Three major types of SOD differ mainly in their prosthetic metals: Cu/Zn, Mn, and Fe. Plants usually have a Cu/ZnSOD in the cytosol, an MnSOD in the mitochondria, and Cu/Zn and/or FeSOD in the chloroplast [105].

Hydrogen peroxide, for the most part, is scavenged by either CAT ($H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$) or peroxidase ($H_2O_2 + AS_2 \rightarrow 2H_2O + AS$). The peroxidative mechanism generally requires a reductant in chloroplasts, and the cytoplasm is ascorbate. The APX is part of the ascorbate–glutathione cycle, which involves successive enzymatic oxidations and reductions of Asa, glutathione and NADP. Enzymatic anti-oxidants, such as SOD, CAT and glutathione peroxidase (GPX) are designed to minimize the concentration of H_2O_2 and superoxide and induced by different stress [106].

Cordeiro et al. [72], using a specific fluorescent dye for cell H_2O_2 detection, observed that root maize seedlings treated with HA increased their ROS content, stimulating gene expression of CAT, thus resulting in increased activity of this enzyme and minimizing the oxidative effect of ROS. The induction of ROS production and the consequent enhancement of enzymatic anti-oxidant metabolism by HA were further observed by García et al. [16]. Aguiar et al. [103] submitted sugarcane to water restriction and, immediately after, observed during the rehydration period that the activity of anti-oxidant enzymes CAT, SOD and APX remained higher in leaf and root tissues of HA-treated plants, when compared to the control

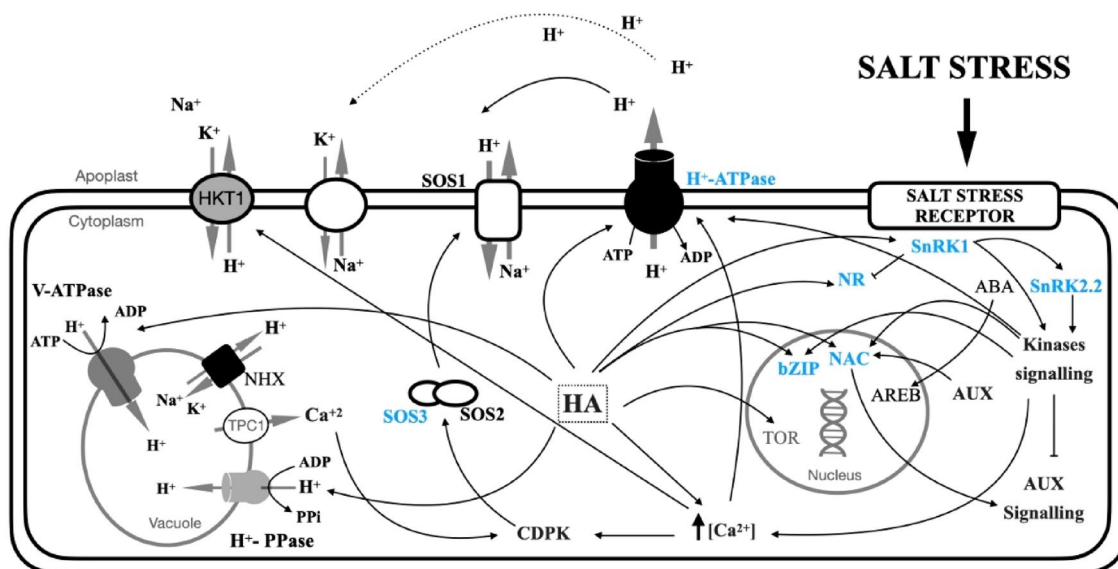


Fig. 1 NaCl stress-signaling pathways and interactions with humic acids. Enzymes or transcripts in cyan were evaluated by Souza et al. The influence of HA on V-ATPase and H-PPase was described by Zandonadi et al. [77] and Ca²⁺ (cyt) pulse, voltage gate Ca²⁺ channel and Ca²⁺-dependent protein kinases (CDPK) activity by Ramos et al. [76]. The HKT1 transporter was evaluated by Khaleda et al. [111], and TOR expression by Trevisan et al. [112] and Canellas et al. [113]. Figure 1 was adapted from reference 117

plants. Humic acids can protect lipids from peroxidation, one of the most dangerous ROS cell effects, by activating an anti-oxidant enzyme protection system [75]. Under salt stress conditions, applying HA increased antioxidant enzyme activities. Foliar and soil + foliar application increased SOD and glutathione reductase (GR) activities, and decreased CAT and APX activities in beans [107].

Ion homeostasis and membrane transport

The intracellular ion homeostasis disruption causes oxidative stress in plants. Maintaining ion homeostasis requires an electrophysiological adjustment that can be sustained by plasma membrane (PM) proton pump stimulation. Figure 1 summarizes the ion homeostasis and the modifications on membrane transport induced by HA, shown by Souza et al. [108]. Activation of PM H⁺-ATPase by HP improves the electrochemical proton gradient that drives ion transport across cell membranes [109], modulating the cellular electrical environment and ion fluxes [76, 110]. According to Khaleda et al. [111], Na⁺ can be removed from cytosol by efflux systems, such as Na⁺/H⁺ antiporters, which transport Na⁺ across the PM and the Salt-Overly Sensitive (SOS) pathway. Na⁺ that enters the root cell and is transported to leaf tissue must be compartmentalized in the vacuole to avoid cytosolic accumulation. This process is mediated by the vacuolar Na⁺/H⁺ antiporter, NHX, which moves Na⁺ into the vacuole in exchange for H⁺.

High-affinity potassium transporter (HKT) family members recover Na⁺ from the xylem to reduce its

transport or accumulation in the shoot. It has also been reported that HA promoted the activity of HKT1 transporters helping *Arabidopsis* to survive salt effects [111]. However, a large part of the machinery responsible for ion homeostasis is recruited by the HA treatment even without the absence of Na⁺ at toxic levels [108].

Hormonal balance

The ROS production and signaling are integrated with the action of auxin (AUX), brassinosteroids (BRA), gibberellins (GIB), ABA, ethylene (ET), strigolactones (SLS), salicylic acid (SA) and jasmonic acid (JA) in the coordinate regulation of plant growth and stress tolerance [114]. The multiple points of reciprocal control and integration nodes involve Ca²⁺-dependent processes and mitogen-activated protein kinase phosphorylation cascade [114]. Plant hormones are vital in linking gene transcription to stress response. Humic substance's hormone-like activity has been documented in great detail [81]. Molecules released from humic superstructures may then access cell membranes and induce different physiological responses, such as hormone auxins AUX, GIB, CK (cytokinins), alkamides (ALK), nitric oxide (NO), ABA and ET [115–123]. However, it is possible that, in addition to the presence of chemical homologues to plant hormones present in the more than 10,000 molecules in the humic supramolecules, the exogenous application of HS changes the plant hormonal balance, by acting as a key regulatory hub in plant responses, integrating hormonal signaling and response pathways.

Stress-induced changes in growth are regulated by phytohormones, such as ABA and ET, which control ROS production and function through synergistic or antagonistic interactions [64]. Despite the relatively large number of research reporting the hormonal effect of HP, there is a gap between the analysis of the hormone balance induced by HP and the anti-stress response. For example, AUXs, CKs, Gas, ET, ABA and SLS balance regulate the P starvation response, one of the most limiting nutritional stresses in highly weathered soils. Their interaction with HP was mentioned by Jindo et al. [124]. Olaetxea et al. [125] found significant increases in ABA root concentration in seedlings treated with HA, showing that the HA-mediated enhancement of root hydraulic conductivity and shoot growth depended on ABA signaling pathways. The AUX-like effect is HS's most well-known phytohormonal behavior. It has been examined in the literature for over half a century [126], indicating that abiotic stress can alter AUX metabolism. According to Potters et al. [127], stress can impact various aspects of auxin homeostasis, including AUX redistribution via effects on the expression of PIN genes, which mediate polar auxin transport. Abiotic stresses can also impede AUX transport by altering the pH in the plant apoplast or by altering the concentrations of phenolics, such as quercetin and kaempferol, which can act as endogenous inhibitors of auxin transport. A higher transcriptional level of PIN genes was found in plants treated with HA [110].

Concluding remarks

Stresses in plants caused by salt, drought, temperature and toxic compounds are the reason behind reduced crop yields. Plants respond to these abiotic stresses partly by activating the expression of stress-responsive

genes, increasing tolerance. Based on the literature, it is clear that HS may contribute to plant adaptation to abiotic stresses. How can this happen? We still do not fully understand how this occurs, but the results from research carried out in recent years, it is possible to see clarity in the middle of the fog. We can simplify the responses of plants to HS and abiotic stresses in a typical physiological response that includes increased generation of ROS (1), the promotion of proton pump activities (2) and changes in plant hormonal balance (3). All of these master variables can act as cell signals that induce other secondary messengers, such as changes on $(Ca^{2+})_{cyt}$ (4), promoting a downstream phosphorylation cascade triggered by Ca^{2+} -dependent protein kinases (CDPK) (5), resulting in gene response by activation of TF (6). These steps may be modified by applying HS (Fig. 2).

When it comes to the general response of the plant to stress and the involvement of HS in these adaptations, the primary focus is the maintenance of cellular homeostasis, more specifically, the redox balance in the case of different abiotic stresses that have the generation of ROS in common. Regarding this balance, Lamar [136] draws attention to an interesting aspect:

“HS possess pro-oxidants (i.e., quinone moieties), in addition to anti-oxidants (phenolic hydroxyls) within their chemical structures, which allow them to take part in redox reactions. Thus, the ability of HS to enhance plant growth may, in part, be redox-based and be influenced by the ratio of pro- to anti-oxidants in the HS chemical structure. Pro-oxidant moieties could be involved in ROS production leading to apoplastic oxidative bursts. In contrast, the anti-oxidant moieties (i.e., polyphenolics) could moderate the oxidative burst in addition

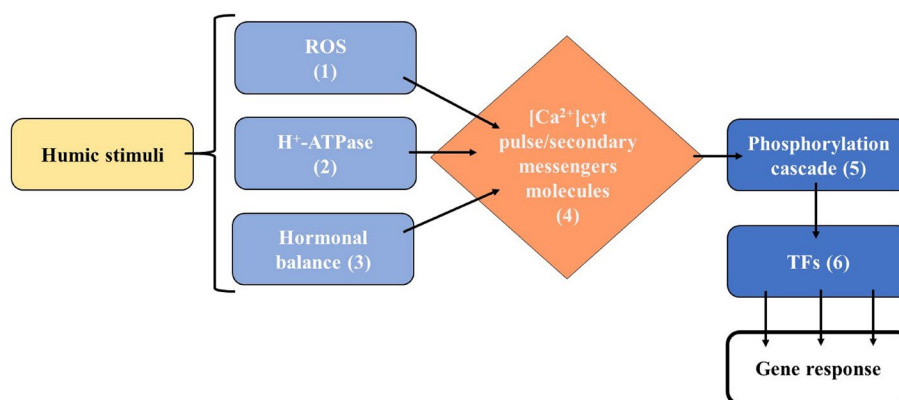


Fig. 2 Humic products can act as elicitors and induce: (1) the production of reactive oxygen in species [16, 72, 74, 75]; (2) activity of proton pumps [128–135] and (3) hormonal activities [80, 81]. The first signals are amplified by secondary messengers, with the calcium pulse in the cytoplasm [4] being among the primary ones [76], triggering a cascade response [5], the most common of which being phosphorylation of proteins that activate transcription factors (TF, 6)

to the plant upregulating its anti-oxidant system to counterbalance the overproduction of ROS and re-establish redox homeostasis, protection of membranes, proteins and nucleic acids resulting in stress tolerance or enhanced growth and productivity”.

The determination of the mode of action and the efficiency of HS as a plant-growth promoter is rendered difficult due to the complex nature of their components. The HS have multiple biological activities, affecting various metabolic processes simultaneously. The chemical nature of HS was unveiled by Piccolo and collaborators, describing it as the supramolecular association of thousands of molecules held together by predominantly weak and hydrophobic forces. The total dissection of humic composition was assessed by the sequential chemical fractionation proposed by Nebbioso and Piccolo [137], named as “humeomic”. If the inventory of each molecular component allows detailed knowledge about the composition, the biological activity seems to result from the interaction between them in a solution. In a seminal work [138], Piccolo indicates that the reactivity of HS in the environment is a product of the acid functional groups (OH and COOH) and of the balance between the amount of hydrophilic and hydrophobic components. Spectroscopic methods such as nuclear magnetic resonance and infrared can obtain these characteristics. The lateral root induction and activity of PM H⁺-ATPase were significantly correlated with the hydrophobic/hydrophilic carbon ratio [139, 140]. In addition, the progressive removal of the humic components using sequential chemical fractionation showed that, when strongly bound components were removed by breaking the ester and ether bonds, the humic residues lost their ability to induce the like-auxin activity (DR5::GUS) and lateral root emergence [141].

However, these capacities were retained in the free or weakly bound molecules [141]. These findings confirm that auxin-like activity in HP is associated with complex hydrophobic structures. The control of these processes seems to be regulated by root exudation, which is significantly larger in plants treated with HA [142]. This process is typical of interaction between complex systems, in which the components interact producing new reactions that encourage the release of bioactive compounds to plant use and the thermodynamic stabilization of the suprastructure. Other characteristics can be used to map the relationship between HS and plant traits, such as Lamar’s electron shuttling capacity [136]. In this way, the bioactivity of HS can be put in the perspective of Yakhin et al. [143], who defined a biostimulant as a product of biological origin that

improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents and not as a sole consequence of the presence of essential plant nutrients, plant growth regulators, or plant protective compounds. This may be considered odd by those who are used to describing biological mechanisms as machines that achieve specific targets and goals.

The concept of emergence, introduced by the definition of Yakhin et al. [143], was questioned by du Jardin et al. [144], who proposed the following issue:

“What do we know about the underlying mechanisms of action and how relevant are the concepts of emergence and interaction to explain biostimulation of plants? Little experimental evidence is available to address this question.”

According to du Jardin et al. [144]:

“Data should be generated which would demonstrate that a plant biostimulant product is a holistic and unitary system of molecules, i.e. that the biostimulatory effect cannot be reproduced by any possible combination of its constituents unless it reconstitutes the complete mixture.”

Data from research [145] showed that the simplification of chemical complexity did not represent a loss of bioactivity until an indefinable chemical level. In other words, in the 1980s and 1990s, the pharmacological approach using chemical inhibitors did not allow definitive answers; the omics approach from the 2000s shuffled the cards showing a much more holistic response in plants than expected, creating effort to give practical meaning to the paradigm break proposed by Piccolo [138] concerning the conformation of HS in solution. The self-assemble driving force to the supramolecular arrangement of HS is the decrease in the total surface area of small molecules with amphiphilic character exposed to water. This surface decrease releases the water molecules from an energetically unfavorable contact with the hydrophobic part. As a result, a global increase in entropy due to the release of water molecules makes the process thermodynamically favorable.

Spontaneous formation of local order assisted (humic aggregate) by an entropy increase and the formation of more or less spherical compartments in microphases (with internal hydrophobic phase) resemble the dissipative structures originally described by Prigogine [145]. The surfactant behavior of HS promotes the aggregation in water in a spontaneous and initially slow manner, which can become faster over time, since a larger layer on the active surface facilitates the aggregation of newly added components. The humic supra-aggregated in the solution can be considered as dissipative structures and described as islands of order surrounded by a sea

of disorder with the maintenance or even an increase of order at the expense of greater disorder in the surrounding environment. These islands can be modified by mass and energy from the external environment, such as the exudation of low molecular-weight organic acids, as shown by Piccolo [138]. Considering these structures as complex chemical systems operating out of equilibrium, their characterization presents an inherent difficulty. Instead of the classic determination of structure (of which a convincing model has never been reached), the objective is to map relationships and to study patterns, that is, to abandon the quantitative approach to qualitative analysis, to change the perspective from structure to process analysis, from interactions with the environment and with the plant.

In this perspective, the characterization of the relationship between hydrophilic and hydrophobic components [139] makes sense for the evaluation of the environmental reactivity of HS, as originally shown by Piccolo [138]. Reactivity can be understood as a result of the organization process resulting from the aggregation of humic matter in a macroscopic set. They are, therefore, not sensitive to microscopic details. It is a collective state with ordered behavior in large samples but imprecise on a small scale. Large samples make it possible to evaluate emerging phenomena; that is, it is at the macro level that the principles of the supramolecular organization gain relevance; self-organization is a process through which a system comes to exhibit global-scale patterns and structures that emerge from the numerous local interactions between its components. The pattern is an emergent property of the system and not imposed on the system by an outside influence. The constitution of an ordered system through self-organization is understood as a primary tendency of complex systems, in contrast to the former emphasis on the degradation of order associated with the entropy principle. The self-organization of humic matter in supramolecular arrangement requires a review of the humification theory, which has already been done by Piccolo et al. [146, 147].

However, considering the physiological aspects of humic matter and its relevant aspects in mitigating plant abiotic stress damage, many questions can be made. Among them, one could consider the supramolecular arrangement as a set of chemical compounds whose key to accessing the various bioactive compartments would lie with the plant and its system for recognizing the environment. The organization pattern of the humic arrangement, described by the hydrophilic/hydrophobic ratio and the electron shuttling capacity [136], could be used as behavior descriptors of suprahmic properties? A

consistent answer is not available at the time, but the HS remains as a tool used by many farmers to mitigate plant stress.

Abbreviations

NAC	NAM, ATAF1,2 and CUC2
MYB	MYB proto-oncogene
MYC	Proto-oncogene
bZIP	Basic leucine zipper
WRKY	WRKY family
AP2/ERF	APETALA2/Ethylene-responsive element-binding protein family
B3	B3 domain-containing proteins
ARR	Two-component response regulator
REMORIN	Remorin family protein
AUX/indole-3-acetic acid	Auxin/indole-3-acetic acid protein family
ARF	Auxin Response Factor family
12) SUVRS5	Histone-lysine N-methyltransferase
bHLH	Basic Helix-Loop-Helix family
HDAC	Histone deacetylase
PPR	Putative pentatricopeptide repeat-containing proteins
F-box	F-box protein
LSD	Lysine-specific demethylase
PLATZ	Plant AT-rich sequence and zinc-binding protein
CAMTA	Calmodulin-binding transcription activators
Ring-type E3	RING-type E3 ubiquitin transferase
PAT	S-Acyltransferase; 22)
GRAS	GRAS transcription factor family (<i>scarecrow</i>)
CRF	Chromatin remodeling factors
AGO	Argonaute
GTE	Transcription factor group E
MTREF	Mitochondrial transcription termination factor family protein
G-box	G-box protein
MOB	MOB kinase activator-like
UBX2	Ubiquitin regulatory X domain-containing protein 2
SWI/SNF	SWItch/sucrose non-fermentable
EIN3	EIN3-like (EIL) transcription factor family
FREE1	Free domain protein required for endosomal sorting 1
TCP	TCP protein domain
LOB	LOB domain-containing protein
Alba	Alba DNA/RNA-binding protein
SEUSS	Transcriptional corepressor SEUSS
C3H Type	C3H-type transcription factor
GRFs	GRF transcription factor
ABH	Alpha/beta hydrolase
CtBP	C-terminal-binding protein
ALF	Alfin-like transcription factor; 40)
HB	Homeobox transcription factor family

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Author contributions

LPC, JGB and FLO conceived the concept. LPC write the first version of this manuscript. RMS did the search literature and data analysis. All the authors read and approved the final manuscript.

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Declarations**Ethics approval and consent to participate**

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Consent for publication

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Not applicable.

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