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Humic and fulvic acids as biostimulants in horticulture

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ABSTRACT

Maintaining food production for a growing world population without compromising natural resources for future generations represents one of the greatest challenges for agricultural science, even compared with the green revolution in the 20th century. The intensification of agriculture has now reached a critical point whereby the negative impacts derived from this activity are now resulting in irreversible global climate change and loss in many ecosystem services. New approaches to help promote sustainable intensification are therefore required. One potential solution to help in this transition is the use of plant biostimulants based on humic substances. In this review we define humic substances in a horticultural context. Their effects on nutrient uptake and plant metabolism are then discussed and a general schematic model of plant-humic responses is presented. The review also highlights the relationship between the chemical properties of humified matter and its bioactivity with specific reference to the promotion of lateral root growth. Finally, we summarize and critically evaluate experimental data related to the overall effect of humic substances applied to horticultural crops. Current evidence suggests that the biostimulant effects of humic substances are characterized by both structural and physiological changes in roots and shoots related to nutrient uptake, assimilation and distribution (nutrient use efficiency traits). In addition, they can induce shifts in plant primary and secondary metabolism related to abiotic stress tolerance which collectively modulate plant growth as well as promoting fitness. In conclusion, the exogenous application of humic substances within agronomic systems can be used to aid the development of sustainable intensification. As most humic substances used in agriculture are currently derived from non-renewable resources like coal and peat, the promotion of this technology also requires the development of new sustainable sources of humic products (e.g. organic wastes).

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1. Introduction

The world is currently facing the combined challenges of feeding a growing population whilst also protecting the environment and producing renewable sources of energy. Demand for food is expected to increase 2–5 fold by 2030 and food production is predicted to increase by 60% in the coming decades to meet these demands (Clair and Lynch, 2010). In the last century, agricultural intensification was driven by inputs derived from non-renewable energy sources (i.e. synthetic fertilisers). Although this approach greatly enhanced crop yields, these practices have also resulted in a major decline in ecological heritage as a result of deforestation, soil erosion, industrial pollution, declines in surface- and groundwater quality and loss of biodiversity (including genetic erosion). These negative consequences of food production continue to proceed at an alarming rate and show no signs of reducing (Altieri, 2002). In addition, it is widely acknowledged that an increase in agricultural activities will further exacerbate the negative impacts of global climate change leading to greater uncertainty in food security (Tilman et al., 2011). Current unsustainable farming practices therefore need to be reviewed since current models of agricultural intensification are neither socially or environmentally sustainable.

The new challenge is to build systems of food production based on alternative intensification strategies (termed "ecological intensification") which promote nutrient-use efficiency, reduce the need for disease and pest control, increase water-use efficiency and

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conservation, and which restore soil fertility (Tittonell, 2014). Ecological intensification aims to reduce the reliance on external inputs while maintaining high productivity levels (Tilman et al., 2011). Within this context, humic substance-based products may provide a potential technology to integrate different biotechnological approaches for ecological intensification related to both promoting plant growth and plant adaptation to new ways of food production.

Humic substances (HS) are formed by chemical and biological transformations of plant and animal matter and from microbial metabolism, and represent the major pool of organic carbon at the earth's surface. They contribute to the regulation of many crucial ecological and environmental processes. For example, HS sustain plant growth and terrestrial life in general, regulate both soil carbon and nitrogen cycling, the growth of plants and microorganisms, the fate and transport of anthropogenic-derived compounds and heavy metals, and the stabilization of soil structure (Piccolo, 1996). A major breakthrough in understanding humus chemistry in the last decade has come with the recognition that humus is a self-assembled supramolecular associations of small heterogeneous molecules held together mainly by weak hydrophobic linkages (Piccolo, 2002). In solution, HS are better depicted as a collection of diverse, relatively low molecular mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds. The hydrophilic/hydrophobic ratio governs its environmental reactivity (Piccolo, 2012).

According to Hayes (2006), the operationally-defined fractionation of humic substances is based on their solubility and was first introduced by Sprengel in 1837. Soil scientists define humic acids (HA) as humus materials that are soluble in aqueous alkaline solutions but precipitate when the pH is adjusted to 1–2. In contrast, fulvic acids (FA) remain in solution after the aqueous alkaline extracts are acidified. This classical definition persists in the older scientific literature but chemically HS are nothing more than a product of a saponification reaction by alkaline extraction from soils and sediments. Piccolo (2002) redefined FA as associations of small hydrophilic molecules in which there are enough acid functional groups to keep the fulvic clusters dispersed in solution at any pH, while humic acids are made of associations of predominantly hydrophobic compounds (polymethylenic chains, fatty acids, steroids compounds) which are stabilized at neutral pH by hydrophobic dispersive forces (van der Walls, π–π, and CH–π bonds). Their conformations grow progressively in size when intermolecular hydrogen bonds are increasingly formed at lower pH until humic matter flocculates.

New formation of intermolecular hydrogen bonding and alteration of pre-existing hydrophobic interactions accounts for the disruption of original supramolecular associations of humic matter. This interpretation implies that water soluble humic associations are mainly stabilized by weak forces and that root-excreted organic acids (typically present in soil solution) may affect the stability of humic conformations and, hence, their effects on plant processes (Piccolo, 2002). This concept suggests that humic molecular complexity may be reduced by the progressive breakage of inter- and intra-molecular interactions that stabilize the complex suprastructures, thus, releasing single humic molecules that can be isolated and identified by combined advanced analytical techniques. This field of analytical chemistry has been termed humeomics (Nebbioso and Piccolo, 2011, 2012; Nebbioso et al., 2015) and allows a holistic evaluation of the chemical constituents of humic assemblies, thus, providing the basis for identifying HS that influence plant performance. For example, the effects of HS on specific plant metabolic processes can now be better understood through humeomics paving the way for the targeted development of HS biostimulant products for use in agriculture.

The aim of this review is to firstly present the main effects of soluble humic matter on plant growth and metabolism and to describe the relationship between its chemical properties and biological action in a structural-activity model. Secondly, we will report on the mechanistic effects of biostimulant humic-based products on horticultural crop production.

2. Direct effects of humic substances on plant growth and development

The promotion of plant growth by HS, defined here as biostimulation, is well documented in the literature (Piccolo et al., 1992; Nardi et al., 2002; Chen et al., 2004; Nardi et al., 2009; Canellas and Olivares, 2014). In support of this, Rose et al. (2014) used a random-effects meta-analysis to show that shoot and root dry weights of different plant species increased about 22% in response to the exogenous application of HS. In view of this, it remains important to understand how HS act on plant metabolism in order to support the future development and successful development and deployment of humic-based technologies. In addition, it is also important to highlight that plant responses to HS also appear to be highly dependent on plant species and ontological state, mode and rate of application, source of HS, and finally the prevailing management and environmental conditions (Trevisan et al., 2010a,b).

Overall, the growth response of monocotyledonous to exogenously applied HS appears to be greater than for dicotyledonous plants although the molecular and physiological basis for this difference remains unclear. In addition, plant physiological responses to HS isolated from brown coal (e.g. lignite, leonardite, subbituminous coals) are less than those observed in response to the addition of HS isolated from peat, composts or vermicomposts (Canellas and Olivares, 2014). Although this information is important for maximising the impact of HS under field application, it is also proving essential to understanding the indirect and direct effects of HS on plant growth.

Humic substances comprise more than 60% of the soil organic matter and are the major component of organic fertilizers and are known to contain significant amounts of nutrients (e.g. N and S; Stevenson, 1994). Due to the stabilization of HS in humic–clay aggregates and their intrinsically slow rate of mineralization in soil, they are not however considered appropriate for a direct source of nutrients to plants (i.e. as a replacement for N and P based fertilisers). Enhanced plant growth in response to HS addition therefore appears not to be related to the nutrient content of HS directly but to interactions of HS with plant membrane transporters responsible for nutrient uptake and membrane associated signal transduction cascades which regulate growth and development.

The direct effect of HS on plant growth is as old as the evolution of terrestrial environments. When plants colonized land, they must have faced a powerful selection pressure directed to increase their absorptive surface areas in soil in order to match the enhanced activity of their photosynthetic organs. Interception of light and CO_2 fixation would therefore have to be in equilibrium with nutrient and water uptake from soils. How HS influenced this selection is not yet clear, although a role played by soil humus in regulating plant evolution is highly likely (Canellas and Olivares, 2014).

It is not surprising that most reported beneficial effects of HS on plant growth appear to be related to their positive influence on changes in root architecture. Vegetation, with its associated microbial community, promotes the alteration of soil inorganic components and consequently directly influences soil solution chemistry. Plants alter the rate of mineral weathering mainly by changing pH, by the release of metal complexation agents, and to a lesser extent by altering the redox potential in soil (Lucas, 2001). Fine roots directly modify the pH of the soil surrounding the root (i.e. rhizosphere) by exuding H^+ ions (via the H^+-ATPase) and via the release of organic acids (e.g. citrate, oxalate, malate). Proton-

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pumping across the root plasma membrane (PM) generates the proton motive force that is necessary to promote the active and passive transport of ions and metabolites through the symplastic pathway (Morsomme & Boutry, 2000). The H^+-ATPase is responsible for the hydrolysis of ATP in the cytoplasm leading to the production of H^+ which is excreted into the apoplast. This creates a lower pH outside the cell and sets up an electrochemical potential difference across the PM which is required to drive the secondary transport of ions across the PM into the cell. This H^+ pumping also lowers the pH of the cell wall (apoplast), activates pH-sensitive enzymes and proteins associated with the wall, and initiates cell-wall loosening and extension growth. This is the basis of the acid growth theory (Hager, 2003), that is induced by auxin. Previous reports have revealed that HS can stimulate the H^+-ATPase of PM vesicles isolated from roots of several plants (Nardi et al., 1991; Varanini et al., 1993; Pinton et al., 1999; Nardi et al., 2000). Canellas et al. (2002) showed clear stimulation of the vanadate-sensitive ATPase activity by HS, including the enhancement of expression of this enzyme. The HS-induced overexpression of the major isoform of the maize PM H^+-ATPase (Mha2) was reported by Quaggiotti et al. (2004). According to the acid growth theory, small bioactive exogenous organic molecules (e.g. indoleacetic acid) access cell receptors to trigger cell signalling. The reduced pH in the wall activates cell-wall-loosening enzymes and initiates the cell volume expansion. The active H^+ secretion through the ATPase is compensated by an antiport of cations. This secondary active ions uptake maintains the internal turgor pressure at a constant value during cell elongation. The active H^+ exudation can be compensated by cations, and the acid-buffer can induce cell wall acidification. These support the acid growth theory (Hager, 2003) and is agreement with previous reports suggesting that intrinsic IAA-like molecules are clustered within HA supramolecular arrangements (Muscolo et al., 1998). The existence of more than 240 auxin-like molecules has been described within the literature (Ferro et al., 2006). Some of these auxin-like molecules are likely to be present in HS and be able to access receptors outside or inside the cell. Their pleiotropic effect may thus account for the diversity of root growth and proton pump activation patterns induced by HS. Ruck et al. (1993) described a receptor that binds auxin in PM, whose H^+-ATPase could be activated in maize protoplasts. This is consistent with the acid growth mechanism proposed for HA bioactivity, by which activation of plasma membrane and tonoplast proton pumps becomes orchestrated during lateral root growth (Zandonadi et al., 2007).

The activation of H^+-ATPase may also be mechanistically linked to root hair proliferation, since it was shown that cell growth depends on extracellular acidification (Peters and Felle, 1999). Changes in organic acid exudation by maize induced by the presence of HS has also been observed (Canellas et al., 2008). This is in line with results showing that maize seedlings changed their organic acid exudation profile in response to HA treatments, which, in turn, enhanced acidification by inducing larger H^+-ATPase synthesis and activity (Canellas et al., 2008). Both Ohno et al. (2004) and Tomasi et al. (2009) described a relationship between organic acids and proton exudation. Puglisi et al. (2008) also reported an enhancement of organic acid exudation in maize seedlings following treatment with HS.

A number of studies have shown that PM H^+-ATPase are regulated by phosphorylation–dephosphorylation mechanisms. Schaller and Sussman (1988) indicated that the oat root PM H^+-ATPase was phosphorylated at serine and threonine residues in a Ca^{2+}-dependent manner. Morsomme and Boutry (2000) postulated that a protein kinase was possibly firstly responsible for the rapid phosphorylation of H^+-ATPase, and, then, a Ca^{2+}/calmodulin-dependent protein kinase would become active, depending on the anion activation by phosphokinase. Using an ion-selective vibrating probe system, Ramos et al. (2015) recently provided the first evidence for HS influencing H^+-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 HS. A clear peak of Ca^{2+} influx in the same root zone of H^+ efflux coupled to very large anion exudation was clearly reported (Fig. 1).

These results are in line with the acid growth theory described above and revealed a new view of the impact of HS on plant signalling related to induction of cell signalling via Ca^{2+} waves. Among the possible effects attributed to the cytosolic increase of Ca^{2+} concentration and down-streaming signalling events, we may include a regulatory H^+ efflux activity in the elongation/differentiation root zone. These results suggest a model for cell signalling in roots elicited by HS that is directly linked to nutrient uptake and lateral root emergence and root hairs (Fig. 2). This process alters the redox potential and pH at the root surface, stimulating root growth and nutrient uptake through promotion of secondary transport and over expression of ion transporters. The intense efflux of H^+ into rhizosphere is offset by a Ca^{2+} influx and anion exudation. This event changes the Ca^{2+} concentration in the cytosol and induces the H^+/Ca^{2+} signalling. The Ca^{2+} transporter was found to be overexpressed in the presence of both HS and the calcium-dependent protein kinase (CDPK), responsible for phosphorylation reactions. While the exudation of organic acids and sugars buffer this system to some extent, the cell signalling pathway operates together with changes in primary and secondary plant metabolism (Fig. 2).

Zandonadi et al. (2010) suggested that HS influences nitric oxide (NO) regulation and root hairs growth, indicating that protein kinases (PK) act as targets of NO signaling as well as Ca^{2+} fluxes. On the other hand, it is well known that the PM H^+-ATPase activity is highly regulated by a PK-dependent phosphorylation of its C-terminal auto-inhibitory domain. We have also shown that HS induced a positive modulation of PM H^+-ATPase activity and expression, thereby controlling the H^+ efflux, root surface pH, and consequently triggering modifications in anion fluxes. The promotion of the PM H^+-ATPase appears to induce a pH signal that modulates Ca^{2+} transport by increasing cytosolic free Ca^{2+} concentration. This, in turn, acts as a second messenger though the mediation of a variety of cellular responses, such as CDPK (calcium dependent protein kinase) activities, PM H^+-ATPase regulation by phosphorylation–dephosphorylating mechanism, and anion channel activation. A large anion efflux is activated by HA negative charges, which are built up on the root surface and re-induce H^+-ATPase activity (Fig. 1).

It is assumed that organic acids exuded by plants into the rhizosphere alter the humic aggregations, thus, releasing smaller size-fractions and even single molecules that may reach the cellular receptors on roots surfaces and exert their bioactivity (Canellas et al., 2010). Nardi et al. (2009) summarized these findings and suggested that HS may behave as signalling molecules in the rhizosphere, perhaps releasing phytohormones or eliciting their production at the plant and/or soil biota level. Although transmembrane receptors have yet to be described, plants respond to the presence of HS by modifying their metabolism and exude compounds that may interact with HS in the rhizosphere by the mechanism of suprastructure alteration, thus, resulting in a specific cross-talk phenomenon between live and dead organic matter in the soil matrix. Humic aggregates may thus contain small molecules and fragments which may interact with plant cell receptors when they become released from the inner humic conformations upon the disrupting action of the short chain organic acids commonly exuded by plants (Piccolo, 2002).

We have verified this model with a sequence of independent experiments in the attempt to find a relationship between the HS chemical composition in solution and its effect on root growth

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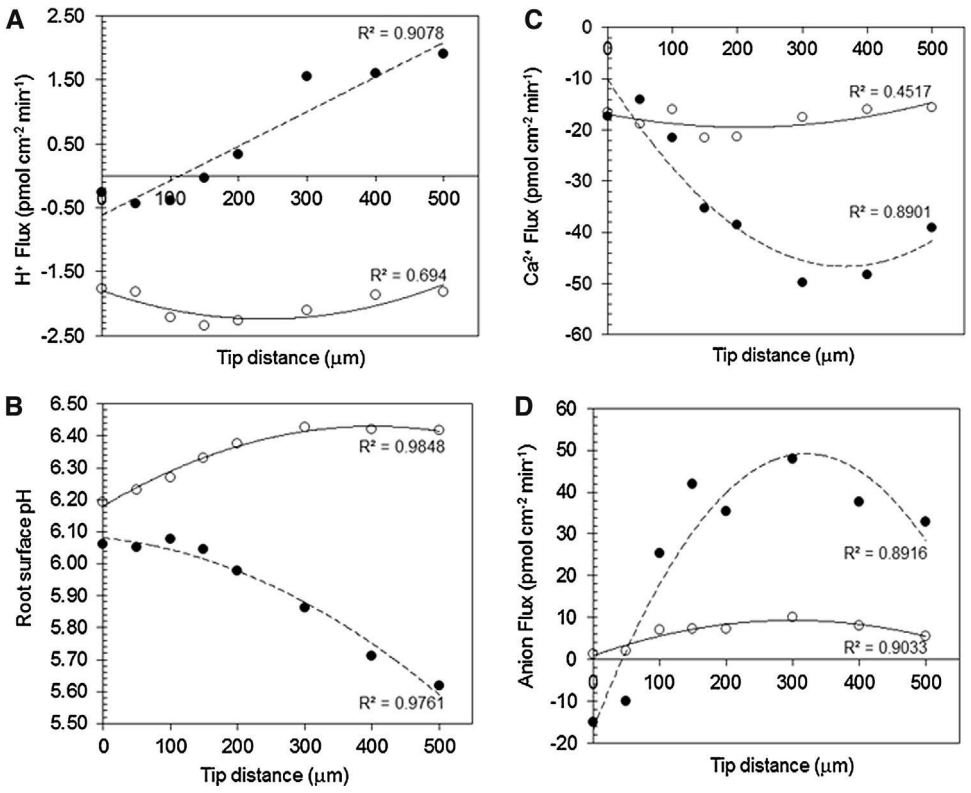


Fig. 1. (A) H^+ influx and (B) Ca^{2+} efflux (C) root surface pH and (D) anion efflux due to rice roots seedling grown in hydroponic culture and supplemented with either 0 mM (○) or 3.5 mM of C humic acids (●). Ion fluxes were analyzed by the vibrating probe system.
Adapted from Ramos et al. (2015).

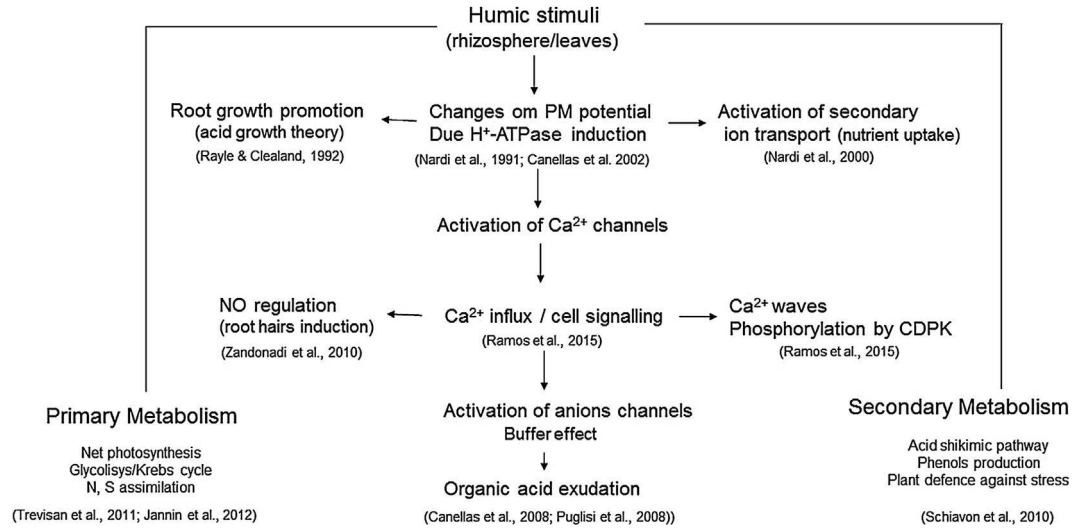


Fig. 2. General picture showing the modes of action of humic substances, in line with the acid growth theory, as elicitor of cell signalling by induction of plasma membrane (PM) H^+-ATPase. NO = nitric oxide; CDPK = calcium-dependent protein kinase.

promotion. We have adopted the number of lateral roots and H^+-ATPase activity as indicators of biostimulation. The first experiment was performed with humic acid (HA) isolated from the surface layer of a soil sequence located in the northern of Rio de Janeiro State, Brazil. This sequence included a wide variety of soils ranging from Brunizens to Alfisols (Canellas et al., 2008a). In this first approach, Pearson correlation was used to relate the main chemical features of HA to root growth promotion. In a second study, only one soil class was used to verify the structure-activity relationship and Oxisols were selected due to their importance as tropical soils and their presence in more than 60% of Brazil. Besides Pearson correlation, we also used a multiple linear regression technique to isolate the properties most related with humic stimulation of root growth (Canellas et al., 2009). By the same mathematical procedure we analysed the results of an experiment where we used humic-like substances isolated from a humified biomass such as a cattle manure vermicompost. Moreover, before the biological assay these humic-like substances were further modified by a series of chemical reactions (oxidation, reduction, hydrolysis, and methylation) to alter the hydrophobic/hydrophilic properties

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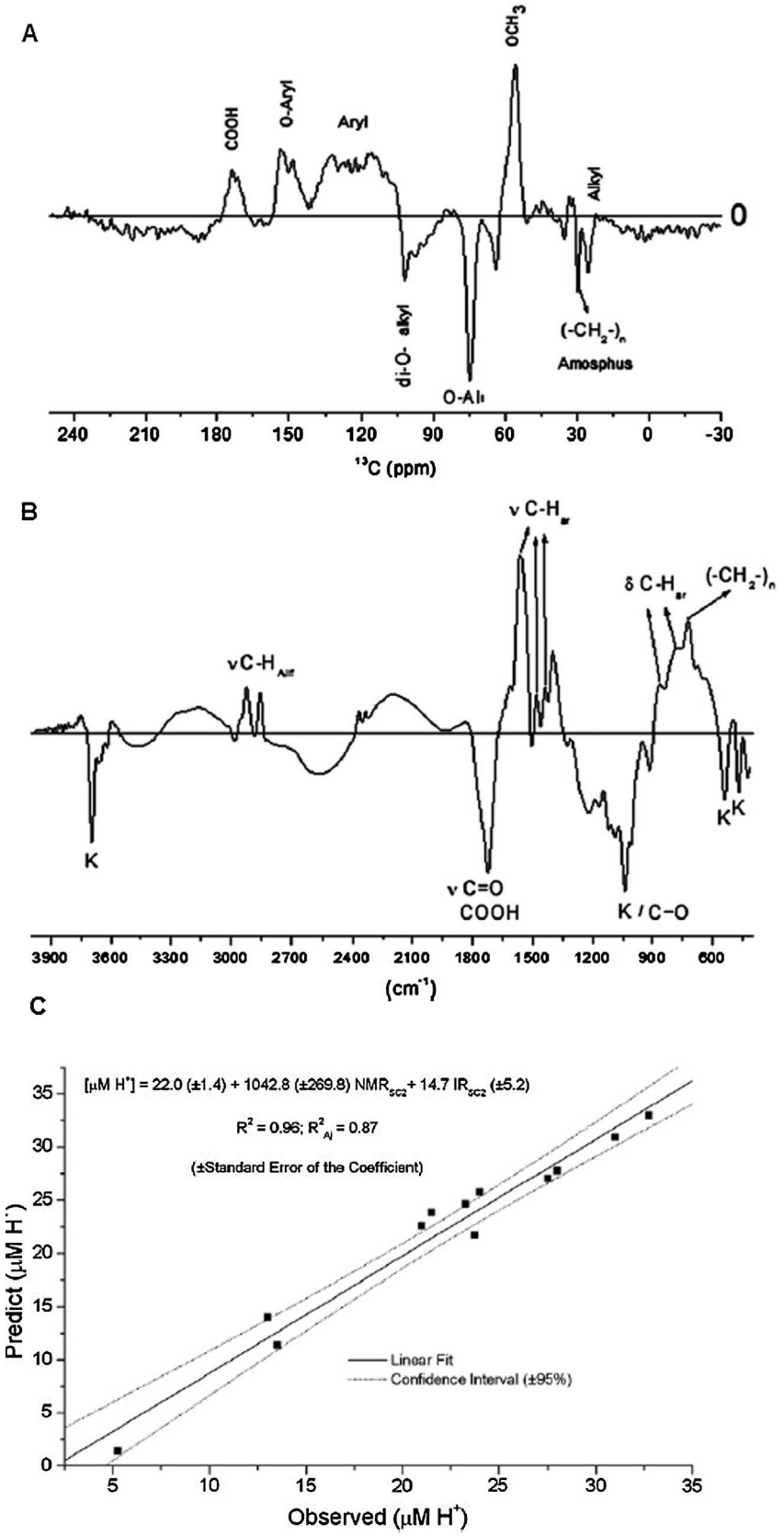


Fig. 3. Relationship between chemical characteristics and bioactivity of humic acids.
Adapted from Aguiar et al. (2013a,b).

of the humic molecular constituents (Dobbss et al., 2010). In all these experiments, we found that the degree of hydrophobicity in HS was the property most related to root growth promotion. These experimental results were then analysed by multivariate methods (principal components and partial linear regression) generating an activity prediction model in which the hydrophobicity also had the highest correlation coefficient (Canellas et al., 2012). Another structure/bioactivity relationship was performed using ^{13}C CPMAS NMR spectra on HA isolated from different vermicomposts and at different maturation times. This exercise provided a model to predict the bioactivity in which methoxylic and aryl groups and carboxylic acids were primarily responsible for humic bioactivity (Fig. 3). Chemical shift related with HA bioactivity showed by NMR spectroscopy include lignin (56 ppm, 124 ppm, 148 and 153 ppm) and COOH groups (174 ppm) and negative loadings for carbohydrates (64, 75 and 102 ppm). For IR- DRIFT the main positive loadings were aryl stretching and bending (1560, 1480, 860 and 780 cm^{-1}) probably from lignins, 2926 cm^{-1} and 2852 cm^{-1} due to C/H asymmetric and symmetric stretching, respectively, probably from long chain fatty acids due to presence of rocking absorption band of $(CH_2)_n$ with $n > 4$ at 720 cm^{-1} and negative loadings due to carbohydrates (around 1100 cm^{-1}, C/O stretching) and carboxylic acids (1724 cm^{-1}, C—O stretching and 1220 cm^{-1}, C/OH stretching and CO/H bending). The predicted values versus measured values for a fitted model produced using CP/MAS ^{13}C NMR and DRIFT data and induction of medium acidification by different humic materials on maize seedlings proved significant (Fig. 3).

The supramolecular nature of humic matter enabled us to devise a new method to identify the molecular structure of humic molecules. In fact, since they are associated in complex conformations held together only by weak bonds, the constituents of HS may be detached from the matrix by a sequence of chemical steps without breaking CC bonds. Nebbioso and Piccolo (2011, 2012) named this process humeomics. It consists of the removal of unbound humic compounds by extraction with organic solvents, followed by a transesterification reaction to break the weakly-bound esters and partitioning the released molecules between water and an organic solvent. The residue is subsequently subjected to an alkaline hydrolysis methanol to liberate humic components from strongly-bound esters, followed by an acidic treatment to disrupt ether bonds and isolate molecules more intimately linked to the humic matrix from either HA extracts and soil humins (Nebbioso and Piccolo, 2011, 2012; Nebbioso et al., 2015). These different humeomic fractions are significantly less complex that the original humic materials (Nebbioso et al., 2014a). They were tested for their capacity to stimulate plant roots (Canellas et al., 2011). It was found that the most inner fractions isolated from strongly-bound esters and ether bonds did not retain the bioactivity shown by the unbound and weakly-bound fractions, thereby showing that the active metabolites were those contained in the most labile fractions. It is interesting to note that the hydrophobic molecules in the unbound and weakly-bound fractions are those of microbial origin, whereas those in fractions more tightly bound to the matrix are still those of untransformed plant origin (Nebbioso et al., 2014b, 2015).

The importance of the hydrophobicity of humic matter in root growth has been demonstrated in a number of studies. Piccolo (1996) postulated that hydrophobic humic components derived from plant degradation and microbial activity are able to randomly incorporate more polar molecules and hence protect them against degradation. Spaccini et al. (2000) proved that biomolecules released in soil during mineralization of maize residues were protected from microbial degradation by surrounding hydrophobic components. Spaccini et al. (2002) further indicated that the process of molecular protection by incorporation into humic hydrophobic domains was more effective with increasing hydrophobicity of protective humic materials. In fact, the supramolecular association of humic molecules held together by weak interactions may be disrupted by the organic acids exuded by plants which fragment the HS into relatively smaller aggregates and a number of unbound humic molecules (Piccolo et al., 1999, 2003; Cozzolino et al., 2001). The molecules released may then access plant cell membranes and elicit different physiological responses. This process agrees with findings showing that maize seedlings changed the profile of exuded organic acids produced in response to HA treatments and that this change was related to an increase of PM H^+-ATPase activity (Canellas et al., 2008b; Canellas et al., 2010). As far as the auxin-like response is considered, the assembling and disassembling behaviour of humic molecular domains upon addition of organic acids appear to explain earlier results on the regulation of this hormone activity by humic matter (Nardi et al., 2002).

Furthermore, it still uncertain whether the apparent molecular size of humic superstructures may be directly associated to the biostimulant properties of HS (Piccolo et al., 1992). Canellas et al. (2008, 2009) and Dobbss et al. (2010) did not find a significant correlation with bioactivity when the apparent molecular weight of different HS was estimated by DOSY ^1H NMR. In support of this, we also carried out an experiment using high performance size exclusion liquid chromatography to obtain different humic fractions with different apparent weight distribution checked by DOSY ^1H NMR. Again no relationship was observed between apparent molecular weight distribution and lateral root growth in maize, tomato or Arabidopsis (Canellas et al., 2010). Muscolo et al. (2007) postulated that the interaction between the root system and humic matter in the rhizosphere is possible when soluble humic molecules are small enough to flow into the apoplastic root compartment and reach the plasma membrane. However, the debate on the biological activity of small over large molecular size has become somewhat obsolete since the assumption that plants can disrupt the humic supramolecular structures through exudation of organic acids.

3. Enhancement of nutrient uptake by humic substances

Humic substances induce H^+-ATPase activity that, in turn, can energize secondary ion transporters and promote nutrient uptake. Nitrate transport across the plasma membrane is facilitated by ion channels, i.e. a secondary active transport, requiring a proton electrochemical gradient generated by induction of the H^+-ATPase (primary transport). The transport of nitrate is thought to be a symporter process involving protons ($2:1H^+:NO_3^-$). Nardi et al. (2000a,b) observed a significant enhancement of nitrate transport (+89% relative to the unamended control) induced by HS. However, Quaggiotti et al. (2004) analysed the expression of ZmNrt2.1, a putative high-affinity nitrate transporter, in roots of maize seedlings treated with HS and did not find any induction in terms of accumulation of transcripts suggesting that the observed increase on nitrate uptake may involve post-transcriptional/post-translational mechanisms of regulation of ZmNrt2.1 and/or an indirect modulation of the uptake process. This latter effect may rely on the action of H^+-ATPases responsible for generating the proton electrochemical difference across the PM essential for nutrient uptake (Serrano, 1993). However, Jannin et al. (2012), working with *Brassica napus* and using q-PCR analysis for the expression of the BnNRT1.1 and BnNRT2.1 genes encoding nitrate transporters, showed an induction of these genes in roots of plants treated with HA. The overexpression was correlated with enhanced nitrate uptake. In the same study, the authors also found an overexpression of BnSultr1.1 and BnSultr1.2 which are PM sulphur transporters.

Formation of soluble HS complexes with micronutrients, i.e. metal–humic complexes, is often reported as a strategy to enhance plant nutrition of trace element (Chen et al., 2004), as metals may be

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prevented from leaching and become more bioavailable to plants (Halim et al., 2003; García-Mina et al., 2004). Aguirre et al. (2009) showed that the expression of genes encoding the Fe(III) chelate-reductase (CsFRO1) and a Fe(II) root transporter (CsIRT1) was also affected by HS. However, the effects were transient and interconnected, although it is possible to demonstrate the capacity of HS to up-regulate some of the main molecular mechanisms (the Fe(III) chelate-reductase/Fe(II) transporter system) involved in Fe root uptake.

4. Effects of humic substances on primary metabolism

Humic substances may promote plant growth through the induction of carbon and nitrogen metabolism. Nitrate reductase (NR), glutamate dehydrogenase (GDH) and glutamine synthetase (GS) are enzymes linked to N assimilation pathways and were stimulated by different HS under a range of conditions (Albuzio et al., 1986; Muscolo et al., 1999; Canellas et al., 2013; Hernandez et al., 2015). A positive dose-dependent effect of HA on activities of the main enzymes involved in the reduction and assimilation of inorganic nitrogen was described by Vaccaro et al. (2015) using semi-quantitative RT-PCR.

Upon HS treatment of crops, Canellas et al. (2013) found a 50% decrease of leaf total carbohydrate content compared to the untreated control plants, and, while glucose and fructose content decreased, starch content enhanced concomitantly. Nardi et al. (2009) reported that HS negatively affected the activity of glucokinase, phosphoglucose isomerase, aldolase, and pyruvate kinase, enzymes involved in glucose metabolism. Invertase activity was enhanced and favoured hydrolysis of sucrose into hexose as substrate available to growing cells (Pizzeghello et al., 2001). When total carbohydrate content as well as reducing sugar decreased following application of humates, it is possible that these metabolites can be used to sustain growth and enhance N metabolism, since enzymes linked to N assimilation were usually stimulated by HS. As a consequence, it was possible to observe high net photosynthesis rates in maize treated with HS (Canellas et al., 2013).

The effect of HS on plant primary metabolism has been challenged by a new biological molecular approach. In this context, Trevisan et al. (2011) concluded from sequence analysis and Gene Ontology classification that a large number of genes involved in developmental and metabolic processes, as well as in transcription regulation or RNA metabolism, were regulated by HS. Using a microarray approach, Jannin et al. (2012) analysed 31,561 genes and found that more than 300 genes were differentially expressed after 3 days of HA treatment, whereas after 30 days of treatment the number of differentially expressed genes was drastically lowered (102 genes in shoots and no differentially expressed genes in roots). In addition, this study indicated that about 50% of genes involved in nitrogen and photosynthetic pathways in shoots were up-regulated. Nitrate reductase, nitrite reductase and the genes involved in amino acid metabolism were found among the genes that are related to nitrogen metabolism. Moreover, 80% of the genes involved in sulfate metabolism were upregulated by HS, with a high representation of genes involved in sulfate uptake and assimilation (sulfate transporter, ATP sulfurylase and serine acetyltransferase). Such clear modifications of primary plant metabolism induced by HS, that were confirmed by molecular biology techniques, may also result in enhanced net photosynthesis rate and field crop production.

5. Effects of humic substances on secondary metabolism and stress alleviation

Besides the significant changes on plant primary metabolism and nutrient uptake, HS may also strongly influence secondary metabolism (Schiavon et al., 2010). These authors showed that HS enhanced the expression of the phenylalanine (tyrosine) ammonia-lyase (PAL/TAL) that catalyses the first main step in the biosynthesis of phenolics, by converting phenylalanine to trans-cinnamic acid and tyrosine to *p*-coumaric acid. The expression of PAL/TAL was accompanied by phenol accumulation in leaves. The authors concluded that the stimulatory effects of HS on plant secondary metabolism provides an innovative approach to explore plant responses to stress. In fact, Olivares et al. (2015) observed a significant enhance of PAL activity in tomato leaves treated with humates isolated from vermicompost and a decrease of the field incidence of *Phytophora infectans*, while Hernandez et al. (2015) observed similar results (enhance on PAL activity) in lettuce.

Other potential applications for HS in horticulture result from its recently described effect on drought stress alleviation. Plants subjected to drought stress showed the capacity to osmotic adjust by maintaining water absorption and cell turgor in response to HS addition (Azevedo and Lea, 2011). Generation of reactive oxygen species (ROS) is potentially harmful under drought stress conditions, since it can induce enzyme inhibition, chlorophyll degradation, and damage to organic molecules, including DNA, and lipid peroxidation (Apel and Hirt, 2004). The peroxidation of lipids is mediated by superoxide radicals (O_2^{2-}), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) that readily attack unsaturated fatty acids, yielding lipid hydroperoxides and alkoxyl (ROd) and peroxyl (ROOd) radicals, that initiate chain reactions in the membranes, changing and disrupting lipid structure, membrane organization and integrity. Several mechanisms are used by plants to scavenge/detoxify ROS (Márquez-García et al., 2011). The importance of enzymatic and non-enzymatic antioxidant defense systems has been shown during drought stress (Kellos et al., 2008). The non-enzymatic antioxidant system comprises compounds such as ascorbate, glutathione, alkaloids, phenols, tocopherols and carotenoids (Gratão et al., 2005). The compounds linked to shikimic pathway (alkaloids, phenols, tocopherols) are stimulated by HA (Schiavon et al., 2010). The enzymatic defense is also induced in the presence of HS.

Peroxidase is a common plant scavenging enzyme involved in regulating oxidative stress. Pizzeghello et al. (2001) found that humic substances increased peroxidase activity from 16 to 270% in comparison to untreated controls, while García et al. (2014) found that HA maintained peroxidase activity below the levels common to plants under stress. However, these authors also found that lipid peroxidation in roots and leaves were lower than in the control, upon treatments with HA. In another study, it was verified that the interaction of HA with the plant radicular system activates an antioxidative enzymatic function, thus controlling the ROS content and modifying the expression of OsTIP by a gene that encodes the tonoplast intrinsic proteins (TIPs) responsible for the direct flow of water and solutes between the cytoplasm and vacuolar compartments (Kaldenhoff and Fischer, 2006). These aquaporins play a fundamental role during intercellular regulation, due to their role in regulating both turgor and osmotic pressure, membrane permeability and cell osmotic balance (Hohmann et al., 2000). Other authors have observed effects of HS on antioxidative defense mechanisms, since they reported stimulation of catalases (CAT) and generation of reactive oxygen species (ROS), that act as intermediaries in plant growth (Cordeiro et al., 2011). Due to these findings, it is not surprising that plants treated with HS showed the best recovery from drought stress (Fig. 4).

Excess quantities of heavy metals in soils represent a common abiotic stress to plants and this has also been shown to be influenced by HS due to their impact on metal speciation and bioavailability (Dumat et al., 2006). Leachability and plant uptake of heavy metals is critically dependent on the mobility and availability of metals in soil, which in turn are affected by the amount of solid

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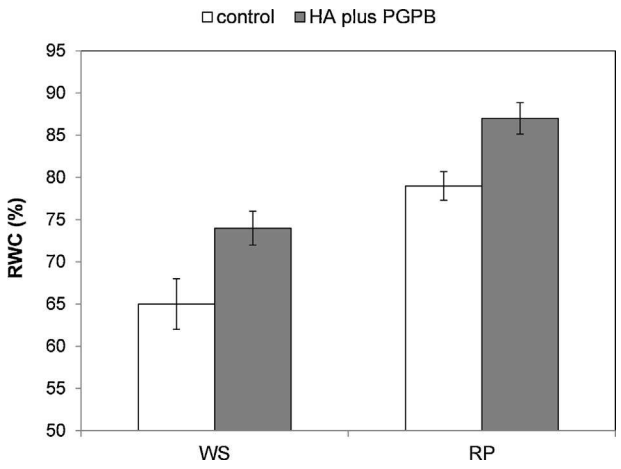


Fig. 4. Relative water content (RWC) of leaves discs from common bean with and without inoculation with humic acids (HA) and plant growth promoting bacteria (PGPB), during water stress (WS) and after recovery period (RP).
Adapted from Melo (2013).

and dissolved organic matter present. The carboxylic and phenolic hydroxyl groups of HS are the main binding sites for metals (Zeng et al., 2002). Shahid et al. (2012) showed that fulvic acids are capable of alleviating Pb phytotoxicity by complexing highly toxic free Pb^{2+} in solution and thus reducing Pb uptake. However, the protective role of fulvic acids against Pb stress depends on the amount applied to the soil with only high concentrations capable of effectively binding Pb and alleviating metal toxicity. HA applied to a contaminated soil was effective in reducing the stress of Pb in corn plants by decreasing translocation of Pb to the shoots (Santos et al., 2014). This implies a greater fixation of the metal in the soil and, consequently, reduction in the risk of transfer into the food chain.

The endogenous plant defence systems could be strengthened by the application of HS. To cope with the negative effects of Cu and Cd, various enzymatic and non-enzymatic antioxidants are also mobilized to quench ROS. After the simultaneous application of HS and heavy metals, the enzyme activities involved in oxidative scavenging (e.g. guaiacol peroxidase, superoxide dismutase and glutathione-S-transferase) were reduced in comparison to the heavy metal-treated plants (Sergiev et al., 2013). Similar results were observed by Haghighi et al. (2010) in which it was observed that HA successfully lowered antioxidant enzyme activity at HA concentrations of 100 mg L^{-1} leading to a decrease in leaf Cd content of Cd treated plants. However, Ouni et al. (2014) reported that depending on the experimental design and the type of metal, both increases and decreases in Cd, Cu and Pb uptake can occur (García-Mina et al., 2004; Inaba and Takenaka, 2005; Kungolos et al., 2006; Montemurro et al., 2008, 2010).

Soil salinity is one of the most important problems in arid and semi-arid regions of the world reducing the yield of wide variety of crops. The mechanisms of growth inhibition include disturbance of plant water retention, due to the high osmotic potential of the external medium and also adverse effects on gas exchange, photosynthesis and protein synthesis (Romero-Aranda et al., 2001). The reclamation of salt-affected soil requires an improvement of physical, chemical biological properties. Under moderate salinity conditions, Cimrin et al. (2010) found that the application of humic acid improved the growth of pepper. Ouni et al. (2014) divided the effect of HS on salt stress into indirect and direct plant effects. The indirect effects of HS are linked to improvements in the physical, chemical and microbiological properties of the soils. The direct actions on plant are due to their effects on germination, plant growth (root and shoot) and hormone-like activity. HS can ameliorate the deleterious effects of salt stress by increasing root growth, altering mineral uptake and decreasing membrane damage, thus inducing salt tolerance. The humic acid application positively affected the yield parameters of plant grown in salinity condition (Türkmen et al., 2004; Paksoy et al., 2010).

6. Application of humic substances in horticulture

Table 1 summarises reports of the biostimulant effects of HS on horticultural crops. The studies were conducted with different HS concentrations and showed a bell-shape curve of dose-response. The optimum concentration was dependent on the specific plant and mode of application (foliar spray or direct soil drench). An increase in nutrient use efficiency was found to be the major biostimulant effect associated with the promotion of vegetable crop growth by HS (Boyhan et al., 2001; Neri et al., 2002; Selim and Mosa, 2012; Naidu et al., 2013; Denre et al., 2014). This was followed by a decrease in the incidence of plant disease (Zaller, 2006; Singh et al., 2010; Naidu et al., 2013; Olivares et al., 2015). Another important aspect was the significant effect on the commercial quality/marketable values of crop products (Boyhan et al., 2001; Neri et al., 2002; Selim and Mosa, 2012; Naidu et al., 2013; Farahi et al., 2013; Denre et al., 2014). In some cases, a better crop response was observed using humic-like substances obtained from compost or vermicompost than from humic extracts from leonardite (the most common commercial source) Azcona et al. (2011), and more by humic acids than by fulvic acids (Lulakis and Petsas, 1995).

Hernandez et al. (2015), using humates as biofertilizers of lettuce production in urban agriculture, observed enhanced nitrate reductase and phenylalanine ammonia lyase activity in leaves. The authors also reported a significant decrease in the length of the production cycle upon humic application, which resulted in a more efficient use of urban space for agriculture; an important factor in the economy of horticultural production.

Baldotto et al. (2009) reported the acceleration of initial growth rates from micropropagated pineapple during the acclimatization stage, especially when plantlets showed difficulty in rooting. After plant transplantation, HS can be useful to help seedlings overcome field stress. The use of HA isolated from vermicompost enhanced tomato seedling growth under greenhouse conditions, and its effect represented an advantage for initial growth and development of transplanted tomato seedlings for field production (Olivares et al., 2015). This stimulation on seedling growth may also become important for plants that show problems of germination (e.g. yellow passion fruit; Cavalcante et al., 2013).

An increase in rooting, growth and early flowering were observed in some studies with ornamental plants (Baldotto et al., 2010, 2012; Baldotto and Baldotto, 2013, 2014), whereby HS application was successful in substituting the more expensive plant growth regulators (Boyhan et al., 2001).

Recently, HS were combined with beneficial microorganisms as plant growth promoter or biological control agents (Siddiqui et al., 2008; Naidu et al., 2013; Olivares et al., 2015). Since HS are considered recalcitrant to microbial activity, it is possible to use them as a carrier to introduce beneficial microorganisms in the field. This new concept of biofertilizer was used for the first time in maize (Canellas et al., 2013) with significant positive effects on crop yield (Canellas et al., 2015). This concept was applied to produce plant growth substrate enriched with HA and plant growth promoting bacteria, and this biologically fortified substrate promoted the enhancement of water soluble P and total nitrogen content (Busato et al., 2012). As described by Olivares et al. (2015), tomato seedlings growing in this fortified substrate were successfully transplanted to the field. Humate and plant growth promoting bacteria applied as substrate to seedling growth, and/or by spraying plant leaves, significantly increased production of tomato during

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Table 1
Effect of humic substances (HS) on vegetables, fruit crops, and ornamental plants.

Crop	Simplified assay description	Effects	References
Apricot	Soil and foliar spray of commercial humic acids at different doses	Increased of yield which ranged from 16 to 33%	Fathy et al. (2010)
Brazilian red-cloak and sanchezia	HA from vermicompost; different doses; pot experiment in greenhouse	Increased by 140% of adventitious rooting	Baldotto and Baldotto (2014)
Broad bean	Humic acid were applied to the seeds	Increased seed germination, yield, nutrient uptake, root weight and length	Akinci et al. (2009)
Broccoli	Commercial liquid HS combined with NPK fertilizers; fertigation; field experiment	Increased 15% of marketable yield and nutrient use efficiency	Selim and Mosa, (2012)
Calathea insignis Hort.	HA combined with biochar; different doses; substrate application; greenhouse experiment	Increased shoot fresh weight, crown breadth and number of leaves by 57%, 45% and 89%, respectively	Zhang et al. (2014)
Chicory	Potassium humate from compost; different doses in soil; greenhouse experiment	Bell shaped dose response curve for shoot fresh and dry weight. Shoot fresh weight increased from 9 to 69%	Valdrighi et al. (1996)
Chrysanthemum	HA from peat combined or not with NPK fertilizers; foliar spray, greenhouse experiment	Dose–effect response curve for shoot and root fresh and dry weight; increased flower diameter by 33%	Fan et al. (2014)
Common beans	HA and FA isolated from charcoal mine used in different concentration with nutrient solution; hydroponic culture; greenhouse experiment	Influenced the kinetic parameters of K uptake. Increased nutrient uptake by 41% on root dry weight	Rosa et al. (2009)
Common beans	Potassium-humate combined with micronutrients and chitosan; foliar spray; field experiment	Increased yield by 25–35%	Ibrahim and Ramadan (2015)
Cucumber	FA from 20 to 2000 ppm added to Hoagland solution	Increased growth and development, nutrient uptake and flowering	Rauthan and Schnitzer (1981)
Croton and hibiscus	HA from vermicompost at different doses; stakes production; greenhouse experiment	Speeded up the rooting of cuttings	Baldotto et al. (2012)
Cymbidium sp	HA from manures at different doses; immersion of cuttings; greenhouse experiment	Decreased the time required for acclimatization; increase in commercial product price due to better standard	Baldotto et al. (2014)
Eggplant	Foliar spray of commercial HA and different N doses; field experiment	Enhanced fruit yield from 23 to 63% and N use efficiency	Azarpour et al. (2012)
Faba bean	Commercial HA plus amino acids were used as foliar treatments in a field experiment	Increased growth and mineral content, 100-seed weight (by 26%) and decreased the damage by chocolate spot and rust diseases	El-Ghamry et al. (2009)
Garlic	HA from peat; different doses; foliar spray; field experiment	Enhanced efficiency of mineral nutrition, and increased from 1.96 to 2.28 fold the pyruvic acid concentration as an indicator of pungency	Denre et al. (2014)
Garlic	Biofertilizer (mix of *Azobacter, Azospirillum* and *Klebsiella*) and 3 sprays with humic acid (rate 2 g/L);field experiment	Increased bulb yield (from 2 to 6%) and quality as well as bulbs storage properties	Abdel-Razzak and El-Sharkawy (2013)
Gladiolus	HA from composts at different doses; corms soaked for 24 h in treatment solutions; greenhouse experiment	Increased growth and promoted early flowering	Baldotto and Baldotto (2013)
Grape	Foliar spray of HA at different plant stages	Increased berry size (width and weight) and improved fruit quality (titratable acidity and soluble solids/titratable acidity)	Ferrara and Brunetti (2010)
Lettuce	HA from vermicompost; foliar spray; field experiment in urban agriculture	Shortened cropping cycle	Hernandez et al. (2015)
Muskmelon	Microbial-enriched compost tea	Enhanced fruit yield (18% of fruit fresh weight), fruit soluble solids, firmness and linear increment in the fruit diameter, decreased powdery mildew incidence and increased nutrient use efficiency	Naidu et al. (2013)
Onion	Solution of commercial HS from leonardite was applied to substrate; greenhouse experiment	Increased root fresh weight by 42–102%	Bettonia et al. (2014)
Onion	Commercial humic acids in solution combined with micronutrients or hormone; field experiment	Increased marketable of bulbs by 26%	Boyhan et al. (2001)
Onion	Field experiment with foliar spray of HA and amino acids at different N doses	Increased total yield by 5–6%	Kandil et al. (2013)
Okra	Compost extracts combined with *Trichoderma*; foliar spray; field experiment	The shoot and tap root length, number of leaves per plant, leaf area were significantly increased; reduced Choanephora wet rot incidence by 76%	Siddiqui et al. (2008)
Peach	Soil and foliar spray of commercial humic acids	Increased fruit yield by 80%	Mansour et al. (2013)
Pepper	HS from compost and leonardite applied to substrate; greenhouse experiment	Increased plant dry matter production by up to 560%; HS from compost with higher bioactivity than those from leonardite	Azcona et al. (2011)
Pepper	Foliar and soil application of HA at different doses; greenhouse experiment	Foliar and soil HA applications led to significantly higher mean fruit weight, and early and total yield than control	Karakurt et al. (2009)

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Table 1 (*Continued*)

Crop	Simplified assay description	Effects	References
Pepper	Soluble humate, pot experiment in greenhouse	Increased N use efficiency and yield by 13%	Varga and Ducsay (2003)
Pineapple	HA from vermicompost combined with beneficial microorganisms; pot filled with soil; greenhouse experiment	Improved growth and adaptation of pineapple plantlets to the ex vitro environment	Baldotto et al. (2010)
Potato	Commercial HA and FA applied to the soil or as foliar spray; field experiment	Enhanced nutrient use efficiency, and tuber weight (by 13%) and decreased incidence of hollow heart	Suh et al. (2014)
Potato	Commercial HA applied to soil at different doses; field experiment	Increased yield from 11% to 22%.	Seyedbagheri (2010)
Strawberry	Vermicompost leachates; foliar spray; field experiment	Increased fruit yield (10–14%) and decreased incidence of grey mould	Singh et al. (2010)
Strawberry	HA commercial soluble product; foliar spray at different doses; hydroponic culture under greenhouse	Enhanced yield (33%), fruit firmness and total soluble solid percent	Farahi et al. (2013)
Strawberry	HS combined with N fertilizers; foliar spray	Enhanced fruit quality reducing the number of misshapen and rotten fruits, and increased the sugar content	Neri et al. (2002)
Strawberry	HA at different doses; foliar spray and fertigation; field experiment	Increased nutrient use efficiency	Ameri and Tehranifar (2012)
Tomato	HA and FA from compost at different doses; hydroponic culture; growth chamber	Doses–response curve for shoot and root growth of tomato seedlings; HA were more bioactive than FA	Lulakis and Petsas (1995)
Tomato and cucumber	HA from vermicompost applied to substrate; different doses; pot greenhouse experiment	Increased tomato and cucumber growth	Atiyeh et al. (2002)
Tomato	HA from vermicompost combined with beneficial microorganisms; substrate application and foliar spray; field experiment	Increased fruit yield by 44–80%; decreased incidence of *Phytophora infestans*	Olivares et al. (2015)
Tomato	Different HA from forest soil mixed with nutrient solution; hydroponic culture	Enhanced net photosynthesis by 68–436% during the vegetative stages and increased fruit sugar content	Haghighi and Teixeira da Silva (2013)
Yellow passion fruit	HA at different doses and times of application as foliar spray	Increased root dry weight by 124% in seedlings	Cavalcante et al. (2013)

HA = humic acids; FA = fulvic acids.

the first year of conversion from conventional to organic farming. Production of tomato in organic farming using HS resulted in relatively high fruit yields (average 84 t ha^{-1}) as compared to the Brazilian production average (70 t ha^{-1}). Production values reached 116 t ha^{-1} when the treatment consisted of humates combined with plant growth promoting bacteria by either (i) inoculation into seedling growth vermicompost substrate, or (ii) foliar sprays within 15 and 30 days after field transplantation. Application of humates and biological enrichment of growth substrate with selected plant growth-promoting bacteria, produced vigorous seedlings in the greenhouse and mature plants and increased growth and yield under field conditions. Further enhancements in plant performance were provided by spraying the products on the leaves. Changes in plant metabolism in response to these treatments were associated with increased fruit production. Therefore, the application of humates isolated from vermicompost in combination with diazotrophic endophytic bacterial inoculation appears to be a powerful biotechnological tool for plant growth promotion in sustainable agriculture systems.

Finally, we would like to mention the study carried out by Hartz and Bottoms (2010) that concluded that HS are ineffective in improving vegetable crop nutrient uptake or productivity. These authors based their criticism on the fact that the main investigations on the physiological effect of HS have been conducted in hydroponic or sand media, while positive responses of the studies under field conditions probably were due to the low organic matter content of soils.

7. Conclusions and future perspectives

HS bioactivity can help to reduce fertilizer application rates, enhance efficiency of nutrient use, replace synthetic plant regulators, improve fruit quality, increase water stress tolerance, decrease disease incidence, enhance early growth and flowering, while their chemical composition may be suitable to behave as carrier to introduce beneficial microorganisms into cropping systems. The use of HS as biostimulants in horticultural crops emerges as a key sustainable technology that could integrate other agricultural practices with the aim of making cropping systems more productive and more efficient, but also having less negative impacts on the environment. In conclusion, there are numerous HS-based formulations which can be applied as biologically active natural products in advanced sustainable agriculture.

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