Hypothetical framework integrating the main mechanisms involved in the promoting action of rhizospheric humic substances on plant root- and shoot-growth

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ABSTRACT

The ability of rhizospheric humic substances to improve plant growth has been well established by many studies carried out using diverse plant species cultivated under many different conditions. These beneficial effects of humic substances on plant development are expressed in both root and shoot. However, the mechanisms responsible for this action of humic substances are only partially known and poorly integrated. In fact, although the studies focused on plant root development are numerous, those dealing with plant shoot development are scarce. Likewise, studies integrating humic effects on root and shoot are also few.

In this context, the main goal of this work is to summarize some of the results regarding the effects of humic substances on plant development within a hypothetical holistic framework that will allow us to interconnect these findings and disclose some features of the functional crosstalk between the effects on soil, root and shoot. Furthermore, the significance of all these mechanisms in plants growing in the field is also discussed.

1. Introduction

A number of studies have shown the strong positive correlation between soil fertility and the soil content of transformed (humified) organic matter, also known as natural organic matter (NOM) (Magdoff and Weil, 2004a). These relationships are mainly related to the significant presence of stable, brown-black, organic fractions in the soil horizons (mainly in the horizon A) where plant roots are developed (rhizosphere) (Pinton et al., 2001a). Moreover, the organic matter in the rhizosphere is highly influenced by and associated with great biodiversity in micro- and macro-organisms (Pinton et al., 2001a,b; Hinsinger et al., 2009).

Although the chemical nature of NOM is controversial, many studies have demonstrated that, in fact, organic fractions in the solid soil interface are chemically and physically linked to clays and other inorganic matrices (metal oxides, metal phosphates and so on…) and minerals (Weil and Magdoff, 2004b; Schumacher et al., 2005; Kinyangi

et al., 2006; Schaumann, 2006a; Lehmann et al., 2008). Moreover, organomineral soil layers contain organic molecules with very different degrees of transformation, from very simple organic molecules present in living matter (sugars, aminoacids, phytoregulators, organic acids, proteins, lignin and cellulosic fragments and so on) to organic fractions with diverse degree of chemically- and/or biologically- induced transformation that have traditionally been related to soil humus (Wershaw, 1993; Stevenson, 1995; Derenne and Largeau, 2001; Weil and Magdoff, 2004b; Piccolo, 2002). Therefore, NOM in soil horizons involves a heterogeneous ensemble of organic molecules with diverse degrees of transformation, with this heterogeneity depending on many soil factors that influence the transformation of fresh organic matter, such as microbiota, temperature, moisture, soil chemical composition and physical texture (Wershaw, 1993; Stevenson, 1995; Derenne and Largeau, 2001; Weil and Magdoff, 2004b; Piccolo, 2002). This molecular complexity is enhanced by the very diverse inorganic fractions that may be bound to organic fractions (Schumacher et al., 2005; Kinyangi et al.,

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Fig. 1. Meeting of the waters of Negro River and Solimoes River in Amazonia.

2006; Schaumann, 2006a; Lehmann et al., 2008).

However, when we focus our attention on one of the NOM fractions that directly interacts with living organisms (microorganisms, plant roots), the object of study is delimited to some extent and the molecular complexity of the system, though still high, might be modelled with greater accuracy. Thus, if we assume that one of the NOM fraction having higher bioactivity is that dissolved in the soil solution and aquatic reservoirs (dissolved organic matter, DOM), the study of the physico-chemical features of DOM fractions in solution will allow us to better understand their effects on plants and microorganisms.

In principle, we can assume that DOM is, to some extent, the product of a natural "extraction" of NOM present in soil resulting from the action of water in soil. This process would be affected by water pH, also related to soil pH. A good example of this "natural" extraction process is the "rio negro" in Amazonia (Fig. 1). As a result of NOM molecular complexity, DOM in soil solution and in aquatic ecosystems will include organic molecules with diverse degrees of chemical and/or biological transformation, from very simple organic molecules (amino acids, sugars, proteins, polysaccharides and so on …) to highly transformed organic molecules (Herbert and Bertsch, 1995; Zsolnay, 1996; Kalbitz, 2001; Deb and Shukla, 2011). These organic molecules may associate with each other in solution, forming more stable molecular assemblies or aggregates, which, in turn, might have new chemical and biological activities. Some authors have concluded that these molecular entities, which traditionally have been named humic substances (HS), resulting from the self-assembly of organic molecules and minerals present in soil solution, can be defined as a type of natural supramolecules (Piccolo, 2002) (Fig. 2). However, this assertion deserves deeper discussion since there are no specific published studies showing that humic molecular aggregates in solution have chemical and biological features qualitatively different from those of their molecular components. In consequence, the question of whether HS are supramolecules or only molecular aggregates remains open and deserves more research. However, some results suggest that some chemical properties probably emerge when humic molecular aggregates are formed, such as amphibility and biological recalcitrance, likely due to the presence of fatty acids and highly hydrophobic molecules in HS molecular aggregates (Chilom and Rice, 2009; Chilom et al., 2013; Khalaf et al., 2014; Nebbioso and Piccolo, 2011; Piccolo, 2002; Terashima et al., 2004). On the other hand, some studies indicate that HS molecular systems include both macromolecules and simpler molecules, which may form large molecular aggregates in solution, with the degree of aggregation depending on the pH, ionic strength and mineral composition of the solution (Garcia-Mina, 2007; Baigorri et al., 2007; Chilom et al., 2009; Esfahani

et al., 2015). In fact, HS molecular aggregates behave like macromolecules from a physico-chemical viewpoint (Garcia-Mina, 2007).

It is obvious that the molecular composition and structural conformation of DOM humic systems (or HS) in solution might be, and probably are, different from those of NOM in solid matrix, although DOM probably also contain significant inorganic residues such as clays, oxides, etc. (Schumacher et al., 2005; Kinyangi et al., 2006; Schaumann, 2006b). However, fluorescence-based studies focused on the molecular conformation in solution of HS extracted from either aquatic or soils ecosystems using various methodologies (reverse osmosis and alkaline extraction), presented very small differences between them (Alberts and Takacs, 2004). This fact indicates, at least, two relevant issues: (i) humic molecular aggregates in alkaline extracts have similar structural features and molecular conformation in solution to those naturally produced in aquatic environments; and (ii) extraction methods do not significantly alter DOM main conformational features. These two points are very important because they show that alkaline extraction is a valuable proxy to study not only DOM reactivity and bioactivity but also some NOM main properties. On the other hand, the fact that the conformational arrangements in solution for HS extracted from the same organic source through different methodologies are similar also indicates that the formation of humic molecular aggregates in solution is not a random process since it seems to be governed by energetically favoured weak molecular forces resulting from the chemical interaction of specific functional groups present in their structure (Piccolo, 2002; Chilom et al., 2013; Esfahani et al., 2015).

On the other hand, the beneficial action of HS treatment on the growth and physiology of plants cultivated in different soil types has been established by many studies (Chen et al., 2004a; Rose et al., 2014; and references therein). Most of these studies describe experiments with HS applied to the roots, but there are also studies involving the application of HS to the leaves (Chen et al., 2004a; Rose et al., 2014). However, the information regarding the specific molecular and physiological mechanisms involved in the plant growth promoting action of HS is rather fragmentary and poorly integrated into a more general overview (Chen et al., 2004a; Rose et al., 2014; Olaetxea et al., 2016).

One relevant point that has to be taken into account is that the beneficial action on plant growth caused by HS applied to the leaves is probably governed by mechanisms different from those involved in the beneficial action resulting from HS application to the roots. For instance, the mechanisms related to nutrient mobilization in soil resulting from the metal complexing ability of HS will not play important roles when HS are applied to the leaves. Likewise, the biochemical processes that may be affected by HS on the leaf surface are probably different, at
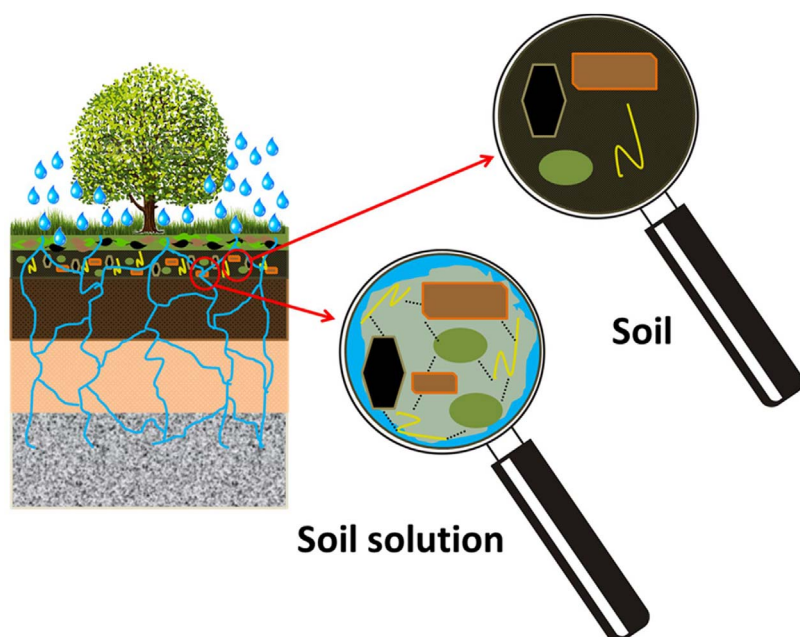
Fig. 2. Schema of dissolved organic matter (DOM) generation from soil natural organic matter (NOM) and water runoff.

least in part, from those triggered by the interaction of HS with roots. Unfortunately, although studies focused on the mechanisms responsible for the effects on plant growth of HS applied to the roots are numerous, those dealing with HS applied to the leaves are scarce and present inconclusive results (Rose et al., 2014, and references therein). In consequence, it becomes clear that specific studies focused on the mechanisms involved in the action of HS applied to the leaves are of great interest. It is true that foliar treatments in open field may also involve some effect on the soil resulting from the humic fractions that do not fall on plant leaves or the run off of HS solution from leaf surface. However, in many cases − for instance, the foliar treatment of maize crops during the cycle − the large leaf surface and plant density make the relevance of this fact negligible. Therefore, explaining the effects of foliar application of HS using mechanisms activated by the HS root application is not justified. In this sense, a probable interaction of HS with soil- and plant-microbiota cannot be ruled out. In fact, the role that the interaction of HS with specific microorganisms either in the rhizosphere or within the plant, might play in the whole mechanism of action of HS on plant growth is a subject of great interest that deserves to be studied in depth.

Another important remark is that most of the studies mentioned and discussed in this article have been carried out under controlled conditions in plants growing in aerated nutrient solution or in inert substrates. Therefore, the real role of these mechanisms in plants growing in the field remains unknown, since the rhizosphere is a very complex system that involves microorganisms, siderophores, root exudates, minerals and DOM-NOM. Therefore, the HS effects on the rhizosphere are probably the result of many biological and chemical processes working together and dependent on soil physicochemical properties, soil microbiota and plant species. Thus, more studies oriented to investigating the role of mechanisms found in plant growing under controlled conditions and without soil, in the beneficial action of HS applied in plants growing in the field are of great and urgent interest.

This review will discuss the mechanisms responsible for the action of HS on plant growth when they are in the soil-root area, which probably are, as mentioned above, different in many respects from those involved in the HS action derived from their application to plant leaves. Likewise, the most important relationships between HS structure and biological activity will also be summarized and discussed.

Finally, taking into account that the HS classification is rather operative, − since it is based on the different solubility of HS main classes in water solutions with different pH values: humic acids (HA), fulvic acids (FA) and humin (Stevenson, 1994); we have complemented this nomenclature with a clear reference to the type of organic material from which HS have been extracted throughout the article (Mora et al., 2014a,b; Garcia et al., 2016a).

## 2. Indirect and direct actions of HS on plant growth

It is quite traditional to divide the HS effects on plant development into indirect and direct effects (Veranini and Pinton, 2001; Garcia et al., 2016a). Indirect effects would be those mediated by the HS action on the rhizosphere (porosity, aggregation-texture, respiration, pool of bioavailable nutrients, microbiota activity and so on …), while direct effects are those linked to the action of HS on plant development resulting from their interaction with cell membranes on the root surface (Fig. 3).

2.1. Indirect HS action on plant development (the "nutritional-environmental"-mediated pathway)

The beneficial actions of HS − either present in native NOM or added to soil as solid organic amendments and liquid formulations − on plant growth, resulting from improvements in plant mineral nutrition, are normally framed within the indirect HS effect class (Chen et al., 2004a). This is because these effects have been linked to the ability of HS to form stable natural complexes (or chelates) with metals, thus increasing the solubility and potential bioavailability of relevant micronutrients (Senesi, 1992; Tipping, 2002; Chen et al., 2004a). On the other hand, recent studies have shown that HS, mainly extracted from soil, leonardite and compost, are able to improve plant mineral nutrition through the activation of the main actors involved in nutrient root uptake and further transport and metabolism within the plant (Quaggiotti et al., 2004; Jannin et al., 2012). These HS-mediated effects were observed at both transcriptional and post-transcriptional levels (nutrient root transporters, proton pumps, and key enzymatic activities) (Quaggiotti et al., 2004; Jannin et al., 2012; Trevisan et al., 2010a, 2011). We refer to these mechanisms as "the complexing pathway" and "the biochemical (molecular) pathway", respectively. The complexing pathway and the biochemical pathway are likely to be complementary to one another, working together in the overall HS action on plant growth.

HS-Indirect effects
Soil rhizosphere mediated effects

Pool of bioavailable nutrients
Texture: porosity, aereation
Water permeation
microbiota
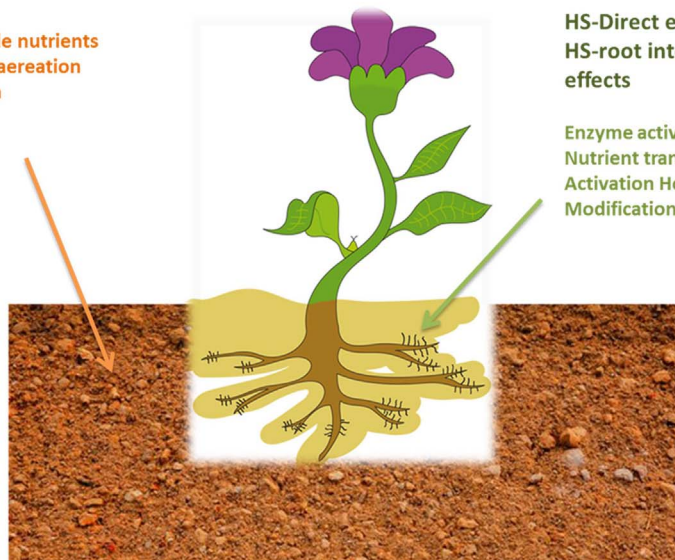
HS-Direct effects
HS-root interaction mediated
effects

Enzyme activities
Nutrient transporters
Activation Hormonal signaling
Modification gene expression

Fig. 3. Direct and Indirect effects of humic substances on plant development.

2.1.1. The complexing pathway

Many studies have shown the ability of HS extracted from diverse sources to increase the concentration of the pool of plant available nutrients in different soil types (Chen et al., 2004a, and references therein). This HS-mediated action was especially important for several micronutrients − mainly Fe, Zn, Mn, and Cu −, as well as some macronutrients such as P (Chen et al., 2004a). Of the different factors probably involved in this action of HS, the most important one seems to be the HS ability to form stable and soluble complexes (or natural chelates) with micronutrients of metallic character, mainly Fe, Cu, Mn and Zn (Chen, 1996). This fact is very important for micronutrient bioavailability in alkaline and calcareous soils where these micronutrients form water insoluble species such as oxides, hydroxides or carbonates (Chen, 1996). Indeed, several studies have shown that more than 80% of metallic micronutrients present in the soil solution of alkaline and calcareous soils are complexed in the DOM humic fraction (Hodgson, 1969; Hodgson et al., 1965, 1966; Geering et al., 1969).

The complexing ability of HS is related to the presence in their structure of oxygen (O) containing functional groups, mainly carboxylic and phenolic groups; Nitrogen (N) −containing functional groups (diverse types of amines and amides); and sulphur (S-)containing functional groups (mainly reduced S-groups); with the ability to form stable chemical bonds with polyvalent metals (M-HA complexes) (Senesi, 1992). In many cases these functional groups are distributed throughout the HS structure forming ligand (binding) sites with singular structural features, such as the salicylic-type binding site, the phthalic-type binding site or the catechol-type binding site (Senesi, 1992) (Fig. 4). Likewise, metal-HA chemical bonds may be intra-molecular or inter-molecular depending on HA and metal concentrations, pH, ionic strength, soil solution composition and temperature (Senesi, 1992).

The main chemical M-HA properties influencing metal bioavailability are their solubility and stability in the soil solution, which in turn are affected by pH and ionic strength (Senesi, 1992; Tipping, 2002; Garcia-Mina et al., 2004).

Many factors can influence the apparent stability constant values of M-HA binding interactions. These factors combine electronic features of both the complexed metal and the complexing HA binding site (Senesi, 1992; Senesi, 1992; Tipping, 2002).

Likewise, both the coordination number and bonding orbital availability have a great influence on the stability of the metal-HA chemical bond, principally for metals included in Group II (Fe, Cu, Zn, Mn, Co and Ni). The order of stability of HA complexes with Group II-metals follows the Irving-Williams series (Senesi, 1992). Metals of Group I (Ca, Mg, K, Na) can also interact with HA complexing sites, but the stability of metal binding is low due to the highly ionic character of the chemical bond (Senesi, 1992).

On the other hand, the electronic features of both the heteroatom (or heteroatoms) present in the HA-binding site and the metal play significant roles in the complexation process (Senesi, 1992; Senesi, 1992; Tipping, 2002). In the case of Fe, highly stable HA complexes mainly involve O-containing groups (carboxylic and phenolic groups), while for Cu, Zn and Ni, N-containing groups (amines) are also involved (Senesi, 1992; Senesi, 1992; Tipping, 2002). Reduced S-containing groups can also be involved in the complexation of some metals such us Cd and Hg (Senesi, 1992). A recent study comparing the stability of Fe (III) and Cu(II) complexation by diverse HS with modified structural domains, indicated that carboxylic acids in aliphatic domains were very important for Fe(III)-HS complexation while phenols and O-alkyl groups in side chains of aromatic moieties were more significant for Cu (II)- HS complexation (Fuentes et al., 2013).

On the other hand, both pH and the M:HA mass ratio influence complex formation and stability (Garcia-Mina, 2006). The pH values favouring binding site ionization (5–9 pH range) would also favour metal complexation. Likewise, low M:HA mass ratios favour highly stable metal binding, since it involves those binding sites with highest metal affinity, whereas high M:HA mass ratios involve metal binding with both high and low stability (Garcia-Mina, 2006) (Fig. 5).

A number of studies have shown that M-HA solubility is largely affected by both pH and M:HA mass ratio (the stoichiometry of the reaction) (Chen, 1996; Chen et al., 2004a; Garcia-Mina, 2006). Assuming that M-HA binding remains stable, acidic pH favours M-HA precipitation due to the protonation of O-containing functional groups, thus decreasing solvation and negative charge repulsion, and favouring inter- and intra-molecular chemical bonds (flocculation) (Garcia-Mina, 2007). Conversely, basic pH favours functional group ionization, thus increasing both solvation and molecular negative charge repulsion, while decreasing inter- and intra-molecular chemical bonds. These processes favour M-HA solubility. This rationale also indicates that pH effects on M-HA solubility are strengthened by the increase in the M:HA mass ratio. Thus, the higher the M:HA mass ratio the lower M-HS solubility (Fig. 5).

In principle, M-HA complexes enhance metal bioavailability when they are soluble and stable in soil solution. Although metal fractions
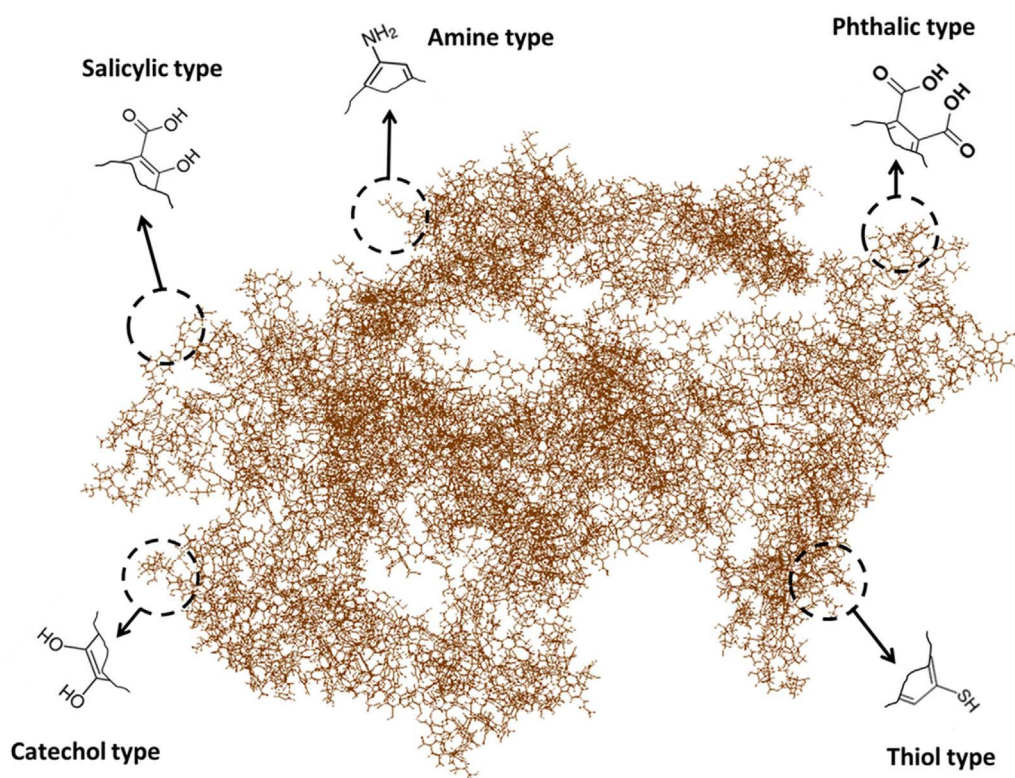
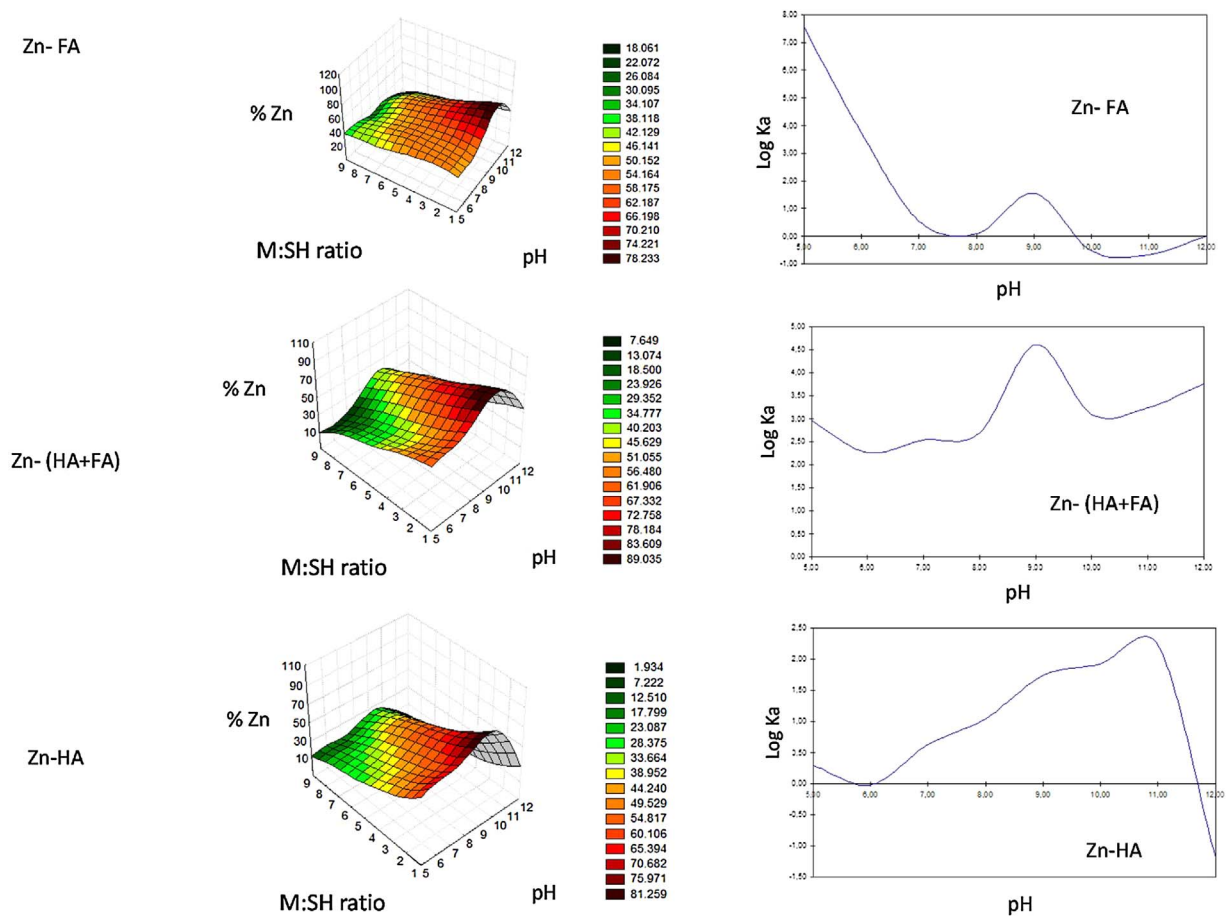**Fig. 4.** Main metal binding sites in humic molecular aggregates.

Fig. 5. Stability and solubility of Zn-Humic complexes in solution as a function of pH and the Zn: humic substances ratio.
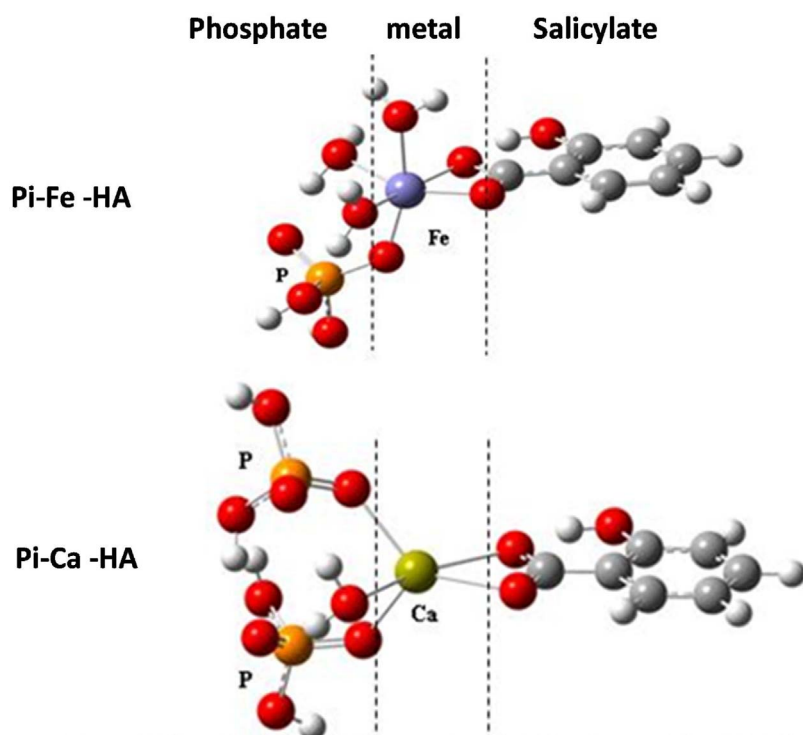
Phosphate   metal   Salicylate

Pi-Fe -HA

Pi-Ca -HA

Fig. 6. Schema of humic-(Fe)(Ca)- Phosphate complexes using salicylic as model binding site in humic structure.

complexed by HA-binding sites with very high metal affinity and stability might be unavailable for plants and microorganisms, numerous studies have shown that, in general, a large fraction of HA complexed metals is fully available for plant root uptake (Garcia-Mina et al., 2004; Chen et al., 2004a,b, and references therein).

Recent studies have also shown that HS metal complexation may also improve the bioavailability of some other nutrients such as P (Gerke, 2010; Erro et al., 2007, 2009, 2012; Urrutia et al., 2013, 2014). Thus, several studies have shown the ability of HS to interact with phosphate through the formation of metal bridges (Urrutia et al., 2013, 2014, and references therein) (Fig. 6). These HS-Metal-Phosphate complexes are stable and soluble under conditions very similar to those of soil solutions (Urrutia et al., 2014). Furthermore, other studies have shown that plants were able to take up both the metal and phosphate from this type of HS complexes, when they were applied to the root in either liquid or solid form (Urrutia et al., 2013, 2014). In this line, some results also suggested that sulphate is probably complexed by HS by a mechanism similar to that involved in phosphate complexation (Baigorri et al., 2013).

In this context, the functional complementarity between plant nutritional needs and functional features of HS is noteworthy, since plants growing in alkaline-calcareous soils prone to suffer micronutrient deficiency can obtain micronutrients from the rhizosphere thanks to the HS ability to form soluble and stable metal complexes at this pH range. Conversely, plants growing in acidic soils with high concentrations of micronutrients, and other metals, in the soil solution may be less affected by micronutrient toxicity thanks to the formation of water insoluble M-HS complexes.

Finally, the fact that the concentration of micronutrients needed by plants is rather low (in the order of mg kg^{-1} of dry matter) implies that the concentration in soil solution of HS required to enhance the transport of the complexed micronutrient to plant roots is also low, and normally it is within the concentration range present in the soil solution of most soil types (Oosterwoud et al., 2010).

2.1.2. The biochemical pathway

2.1.2.1. Macronutrients and secondary nutrients. In numerous experiments carried out in different plant species growing under diverse soil conditions, HS application to either the rhizosphere or the leaves, caused significant improvements in N root uptake and further assimilation within the plant (Rose et al., 2014, and reference therein). Moreover, this enhancement in N root uptake, in this case in nitrate form, was linked to significant increases in the activity of the root plasma membrane H$^+$-ATPase in plants growing in hydroponics (Pinton et al., 1997, 1999a; Santi et al., 2003).

Further studies showed that HS extracted from vermicompost were able to promote the up-regulation of nitrate transporters in maize leaves but not in roots (Quaggiotti et al., 2004). Later, studies carried out using sedimentary humic acids (SHA) obtained from black peat showed that these compounds were able to enhance both nitrate-transporter gene expression and nitrate root uptake, in rapeseed (Jannin et al., 2012). Likewise, studies made in cucumber with SHAs extracted from leonardite reported that the HS- promoting action on shoot growth was associated with an increase in the root to shoot translocation of active cytokinins, which in turn was also linked to an increase in root PM H$^+$-ATPase activity, and nitrate root uptake and further root to shoot translocation (Mora et al., 2010).The latter study is a good example of the functional links between hormonally regulated mechanisms and the HS- mediated beneficial nutritional action in the whole action of HS on plant growth.

On the other hand, several studies have reported that these effects of HS on N root uptake were also linked to increases in the activity of enzymes involved in N metabolism, − such as the nitrate reductase and other enzymes involved in ammonium assimilation −, in several plant species, such as maize (Nardi et al., 2000; Vaccaro et al., 2009), cucumber (Mora et al., 2010), rapeseed (Jannin et al., 2012) or, recently, rice (Tavares et al., 2017).

Many studies have shown the importance of S for plant N use efficiency (Marschner, 1995, 2012). In fact, S deficiency is associated with significant decreases in the activity of many of the main processes involved in (or depending on) N metabolism, such as protein synthesis and photosynthesis (Marschner, 1995, 2012). Moreover, sulphate root uptake is also linked to root PM-H$^+$-ATPase activity as in the case of nitrate (Marschner, 1995, 2012). It is thus possible that HS are able to enhance sulphate root uptake and further assimilation within the plant. This hypothesis was experimentally proved in rapeseed plants whose

roots were treated with peat SHA (Jannin et al., 2012). This study showed that peat HA was able to enhance both sulphate root uptake and the expression of those genes encoding the main sulphate transporters in the root (Jannin et al., 2012).

Several studies have reported that the root application of HS with diverse origin was able to improve overall P plant nutrition (Urrutia et al., 2014, and references therein). Normally, these results have been linked to the HS ability to mobilize and solubilize phosphate from insoluble forms in the rhizosphere (Magdoff and Weil, 2004a,b). However, recent studies have shown that HA obtained from vermicompost was also able to increase phosphate root uptake and modify the gene expression of one of the main high affinity root transporters of phosphate in tomato (LPT2) (Jindo et al., 2016). However, this effect was observed in plants grown under phosphate sufficiency but not under phosphate deficiency (Jindo et al., 2016). This unexpected result needs further research in order to be adequately understood.

Many results showed that the growth promoting action of HS is normally associated with increases in K leaf concentration and root to shoot translocation (Mora et al., 2010; Rose et al., 2014 and references therein). However, the mechanism by which HS improves K root uptake has not yet been studied in depth. It is very probable that the HS-mediated increase in the root PM H^+—ATPase activity might favour K influx into root cells (Arango et al., 2003). Nevertheless, specific studies of the effects of HS on the regulation of the main K channels (aquaporins, PIPs) and high affinity transporters (AKT, HAK and HKT families) are needed in order to clarify the mechanisms involved in the effects of HS on K root uptake and further transport within the plant.

As for Ca and Mg, many studies have shown that the HS application to either the root or the leaves led to increases in the concentration of Ca and Mg in leaves as well as their root to shoot translocation (Mora et al., 2010; Rose et al., 2014). However, there are no specific studies focused on the HS effects on the regulation of Ca and Mg root uptake and further transport at molecular level. It is quite clear that the ability of HS to increase ATPase's activity in roots might play an important role in all this process (Arango et al., 2003). Thus, a recent study has reported that changes in cytoplasmic Ca^{++} concentrations (Ca-waves) may play a very significant role as a secondary messenger for the effect of HS on root growth, through the activation of kinase- mediated protein phosphorylation (Ramos et al., 2015).

2.1.2.2. Micronutrients. Several studies indicated that HS can improve micronutrient plant nutrition not only by increasing their availability in the rhizosphere thanks to their chelating ability, but also by enhancing the root capability to take up those micronutrients from the soil solution (Chen et al., 2004a,b; Aguirre et al., 2009).

A number of studies have shown that Fe deficiency has a significant impact on crop quality and yields (Marschner, 1995, 2012). In fact, plants have evolved sophisticated mechanisms in order to cope with Fe limiting conditions. These special plant responses are expressed in both shoot and root, and vary depending on plant species (Marschner, 1995, 2012). Whereas dicotyledonous plants evolved a strategy based on the reduction of complexed- or chelated- Fe (III) into Fe (II) by inducing a chelate reductase in roots coupled to a specific Fe (II) transporter, as well as rhizosphere acidification, graminaceous plants used a Fe (III) chelating strategy based on the biosynthesis and release to the rhizosphere of Fe(III) chelating compounds named phytosiderophores (Marschner, 1995, 2012). It has been well established that HS improves Fe availability for plants growing in soils prone to Fe deficiency, mainly alkaline and calcareous soils (Chen, 1996; Chen, 1996; Chen et al., 2004 a,b). This beneficial action of HS is associated with their ability to complex Fe, thus increasing its solubility in soil solution (Chen, 1996; Chen et al., 2004b). However, other studies have observed that HS might affect iron root uptake via mechanisms related to root functionality (Pinton et al., 1998, 1999b, 2009). Thus, several studies reported that HS-Fe complexes are a better substrate for Fe (III) chelate reductase than other types or Fe(III) chelates such as Fe-EDTA

(ethylenediaminetetra-acetic acid) (Pinton et al., 1999b). Likewise, other experiments showed that a SHA obtained from leonardite was able to activate the main root physiological responses to Fe deficiency at both transcriptional and post-transcriptional levels (chelate reductase, Fe(II) transporter and PM H^+-ATPase), in the roots of cucumber plants cultivated without limitation in Fe availability (Aguirre et al., 2009). Further studies reported that a SHA obtained from black peat was able to promote the up-regulation of the Fe (II) transporter (IRT1) in both the roots and leaves of rapeseed plants (Billard et al., 2014). These results were correlated to significant increases in the Fe concentration in leaves (Billard et al., 2014), and to an improvement in chloroplast efficiency (Billard et al., 2014; Billard et al., 2014; Abros'kin et al., 2016).

In this sense, Garnica et al. (unpublished results) also observed that the application of a leonardite SHA to the roots of wheat plants growing under Fe limiting conditions was able to enhance phytosiderophore root release to the rhizosphere as well as Fe remobilization within the plant.

Finally, genes codifying transporters related to Cu, Mn and Zn root uptake and further root to shoot translocation within the plant, such as COMT2 and NRAMP3, were also up-regulated in rapeseed roots upon the root application of a peat SHA (Billard et al., 2014).

Overall, the above presented results indicate that HS can improve the root uptake and further transport of the main mineral nutrients within the plant by combining indirect effects on soil rhizosphere, mainly by increasing nutrient bioavailability through chelation, with direct effects on plant roots by modifying the gene expression and activity of relevant molecular players involved in nutrient root uptake and metabolism, such as nutrient transporters and specific enzymes (Fig. 7).

It is thus possible that the action of HS on the regulation of the nutrient uptake both at transcriptional and post-transcriptional levels results from an induction of root responses by the presence of very low concentrations of available nutrients in the rhizosphere that are detected by a sensing system in roots. This signal would trigger the activation of specific root responses to cope with oncoming nutrient deficiency (Marschner, 2012). However, this fact might also be coupled to secondary signals deriving from root hormonal pathways that are activated by HS (Nardi et al., 2002; Olaetxea et al., 2016). In any case, all these findings show that is very likely that the whole action of HS on plant mineral nutrition involves a coordinated functional crosstalk between indirect and direct HS effects on the soil-plant system.

2.2. Direct action of HS on plant roots and shoots at transcriptional and post-transcriptional levels and its consequences for plant physiology and development

Many studies have shown that HS affect plant metabolism and physiology (Vaughan and Malcolm, 1985; Nardi et al., 2002; Rose et al., 2014; Berbara and Garcia, 2004; Olaetxea et al., 2016). This action has been referred to as "direct effects" since they occurred in plant growing under optimum conditions and could not be explained as a result of HS action on the substrate, nutrient solution or soil (Nardi et al., 2002; Rose et al., 2014 and references therein). However, in this case the HS concentrations needed to cause plant biostimulation were much higher than those involved in the improvement of plant mineral nutrition (Chen and Aviad, 1990; Chen et al., 2004a; Rose et al., 2014). While the presence of 2–50 mg L^{-1} in soil solution of HS was enough to improve micronutrient plant nutrition, 150–350 mg/ L^{-1} in soil solution of HS was needed to cause consistent plant biostimulation (Chen and Aviad, 1990; Chen et al., 2004a; Garcia-Mina et al., 2004; Rose et al., 2014). In consequence, in the case of many natural and intact ecosystems where the HS concentration in soil solution is rather low (less than 50 mg L^{-1}), the main role of HS would be to provide bioavailable mineral nutrients for plants and microorganisms. However, in agricultural ecosystems (for instance, fertirrigated crops), where farmers can localize liquid products into the root zone through drip irrigation systems, − thus increasing HS concentration in the rhizosphere −, HS may
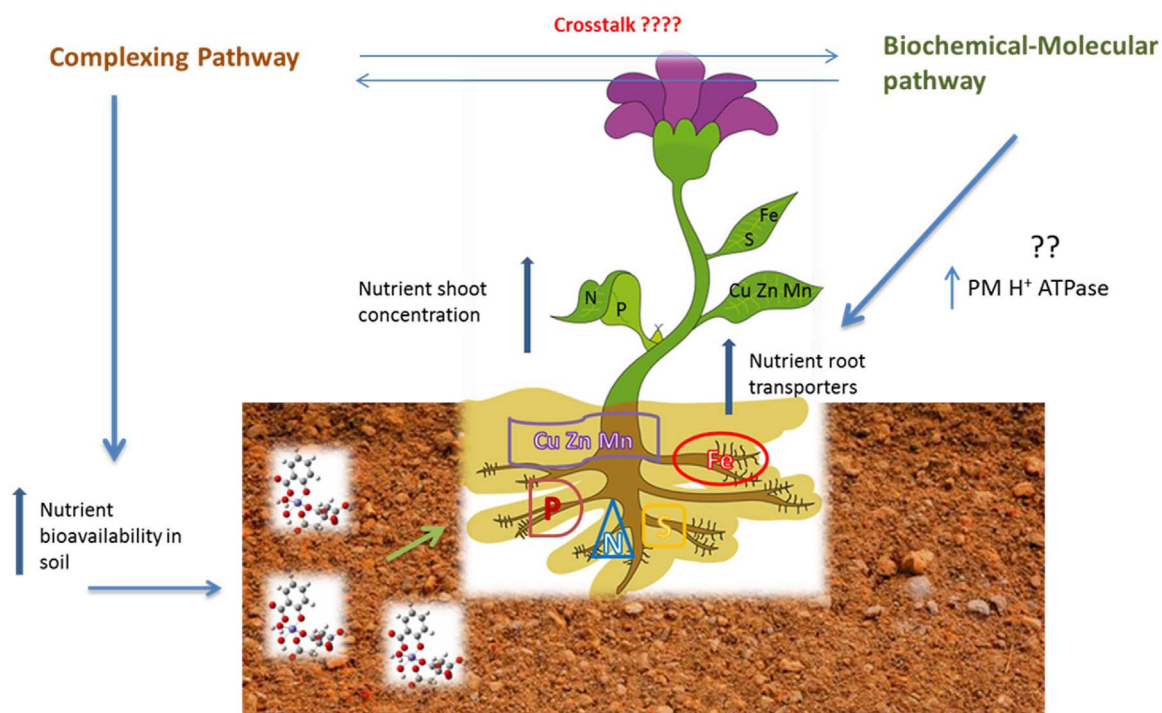
HS-mediated plant nutrition improvement

Fig. 7. Involvement of the complexing pathway and the biochemical pathway in the beneficial effects of humic substances on plant mineral nutrition.

affect plant growth via two main types of effects: (i) improving soil features and nutrient availability (indirect effects), and (ii) interacting directly with cell walls on the root surface (direct effects).

2.2.1. Action of HS on the growth and functionality of plant roots

A number of studies have shown that both SHS and CHS are capable of promoting root growth and modifying root architecture in different plant species (Vaughan and Malcolm, 1985; Nardi et al., 2002; Rose et al., 2014). In general HS affect the whole root growth (expressed by dry matter production) as well as the proliferation of adventitious-lateral roots and root hairs (Canellas et al., 2002; Trevisan et al., 2010a,b).

Numerous studies have shown that HS obtained from compost and organic sediments were able to induce lateral root proliferation probably by activating signalling pathways regulated by auxin and nitric oxide in roots (Trevisan et al., 2010a,b; Zandonadi et al., 2007, 2010; Mora et al., 2012). In fact these HS effects were accompanied by the up-regulation of several auxin regulated genes in roots (Trevisan et al., 2010b). Likewise, the increase in IAA and NO root concentrations was essential for the HS-mediated enhancement of root PM-H$^+$ ATPase activity (Zandonadi et al., 2007, 2010). Other experiments made using a leonardite SHA showed that these effects on IAA and NO were also coupled with increases in ethylene root production and ABA root concentration (Mora et al., 2012, 2014a,b).

It is known that ABA and auxins can work in an integrated pathway, co-regulating several signalling pathways at root level. A loss of function in ABA-insensitive mutants results in decreased emission of lateral roots (Brady et al., 2003). ABA receptors (PYL8 and PYL9) are known to function as crosstalk nodes between ABA and auxins, and their signal is transduced by a group of transcription factors in order to regulate lateral roots (Xing et al., 2016). Overexpression of the ABA-insensitive 4 transcription factor also impairs lateral root formation by reducing the expression of the auxin transporter PINFORMED 1 (Inbar-Shkolnik and Bar-Zvi, 2010). This complex hormonal crosstalk can be extended to both NO and ethylene (Simontacchi et al., 2013).

Although the effects of HS on root lateral proliferation can be explained by its effect on auxin, NO and ethylene signalling pathways (Zandonadi et al., 2007, 2010), the increase in the whole root growth (reflected in the root dry matter production) seems to be expressed by signalling pathways independent of these three hormones (IAA, NO and ethylene) (Mora et al., 2012). In consequence, other molecular signals must be involved in the action of HS on whole root growth.

Recent studies have reported that HS also regulate the reactive oxygen species (ROS) content at the cellular level as well as the expression of responsive superoxide dismutase (SOD) genes in the cytosol (cCu/Zn-SOD1 and cCu/Zn-SOD2) (Garcia et al., 2016a). This HS-mediated action on ROS (H$_2$O$_2$ and O$_2^-$) regulation and control occurs in the root regions corresponding to cell elongation and differentiation (García et al., 2016a). These results, which are in line with previous studies (Muscolo et al., 1993; Muscolo and Sidari, 2009), suggested a potential role of ROS in the HS-mediated action on root development. A number of studies have indicated the signalling role of ROS in the regulation of lateral root formation, membrane polarization and Ca^{2+} signalling (Foreman et al., 2003, Demidchik et al., 2007, 2009). An increase of H$_2$O$_2$ in the cytosol may induce hyperpolarization of Ca^{2+} channels in the cell elongation region (Demidchik et al., 2007) and lead to NADPH oxidase stimulation and induction of MAP kinase 3 transcription (Demidchik et al., 2009). In fact, this type of action of HS on both Ca-signalling pathways and protein phosphorylation is said to be involved in the whole post-transcriptional action of HS on root growth (Ramos et al., 2015).

In this framework, it is thus possible that ROS might be a very important signal for the whole action of HS on root growth and architecture, either integrated into other hormonal pathways or acting alone (Fig. 8).

2.2.2. Action of HS on shoot growth

Although the main signalling pathways involved in the HS-mediated action on root growth and functionality have been extensively studied, the mechanisms responsible for the HS action promoting shoot growth as well as the relationships between HS-mediated shoot growth and HS
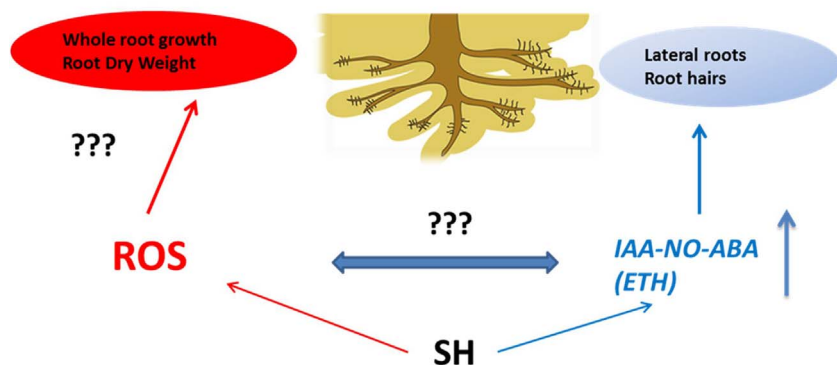
Root Development

effects in roots are less known.

A number of studies have shown that the signal action of nitrate increasing shoot growth was coupled with a promotion of the conversion of inactive cytokinins forms in active ones, along with an enhancement of the root to shoot translocation of active cytokinins (Sakakibara, 2003; Sakakibara et al., 2006; Rahayu et al., 2005; Garnica et al., 2010).

On the other hand, as described above, the increase of root PM H$^+$ ATPase activity caused by SHA root application was associated with significant increases in nitrate root uptake in several plant species. In this context, it is plausible that the mechanism responsible for HS-mediated enhancement of shoot growth involves crosstalk between nitrate and cytokinin signalling pathways. This hypothesis was supported by further studies showing that the shoot promotion caused by a leonardite SHA was coupled with an increase in both root PM-H$^+$ ATPase activity and nitrate root uptake, which in turn was also linked to a concomitant increase in the root to shoot translocation of active cytokinins (Mora et al., 2010). Further studies have reported that the SHA-mediated action on shoot growth was also dependent on the increase in IAA and NO root concentrations caused by SHA in cucumber, since the root application of inhibitors of the function of both IAA and NO prevented the shoot promoting action of SHA (Mora et al., 2014a,b). This study also showed that the increase in ABA root concentration mediated by SHA was dependent on both IAA and NO (Mora et al., 2014a,b).

In line with these results, Olaetxea et al. (2015) have shown that the shoot growth promotion mediated by a leonardite SHA was functionally dependent on an increase in root hydraulic conductivity, which in turn was dependent on ABA signalling pathways. This study also reported the leonardite SHA-mediated enhancement of shoot growth probably involves ABA-dependent pathways that regulate plasma membrane aquaporin activity in roots (Olaetxea et al., 2015).

Taken together, these results indicate that HS promote shoot growth through signalling pathways regulated by the effects of SHA on IAA-, NO- and ABA- signalling pathways (Fig. 9).

2.2.3. HS-mediated improvement of the growth of plants subjected to stress

Several studies have shown that HS significantly improve plant resistance to abiotic and, to lesser extent, biotic stresses (Polák and Pospíšil, 1995; Berbara and García, 2014; García et al., 2012, 2014, 2016a; Aguiar et al., 2016). This HS ability was also coupled with significant changes in the biosynthesis and concentration of secondary metabolites directly involved in stress alleviation, such as proline (abiotic stress) and phenolic compounds (phytoalexins) (biotic stress) (Polák and Pospíšil, 1995; Berbara and García, 2014; García et al.,

2016a; Aguiar et al., 2016) (Fig. 10). These results are also in line with the fact that HS root application significantly affected the expression of genes encoding enzymes and transporters involved in plant responses to abiotic and biotic stresses (Trevisan et al., 2011; Jannin et al., 2012). Furthermore, considering the relevant role of the regulation of ROS accumulation in the control of the damage caused by these types of stresses, these results suggest that the stress alleviation caused by HS probably results from the HS-ability to regulate ROS accumulation and metabolism (Berbara and García, 2014; García et al., 2012, 2014, 2016a; Aguiar et al., 2016) These studies show that the application of HA extracted from vermicompost to the roots of rice plants caused an increase in the concentration of both H$_2$O$_2$ and O$_2^-$ in specific root zones (Fig. 11-2) (Garcia et al., 2016a). This action was accompanied by an increase in the activity and the gene expression of the main enzymes involved in ROS metabolism (Fig. 11-3,4) (Garcia et al., 2016a). These results clearly show how a vermicompost-HA is able to both induce ROS production and regulate ROS concentration, thus suggesting that ROS may work as a signal for the control of root development (Berbara and García, 2014, and references therein; Garcia et al., 2016a). Furthermore, bearing in mind the close functional relationships between ROS, auxin, NO and ABA, it is likely that all these hormones might also be involved in the HS-mediated alleviation of plant stress (Kawano, 2003; Tognetti et al., 2012; Berbara and García, 2014).

In any case, the specific role of ROS into the overall mechanism of action of HS on root development in non-stressed and stressed plants deserves further investigation.

Finally, root ABA − a stress related plant hormone– might also be involved in the beneficial action of HS on plants grown under stress conditions as well as the HS-mediated effects on root development (García et al., 2014; Xu et al., 2013; Liu et al., 2014; Olaetxea et al., 2015). Thus, under moderate osmotic stress, an increase in ABA may increase auxin transport and consequently both H$^+$-ATPase activity and root elongation (Xu et al., 2013; Liu et al., 2014 Liu et al., 2014). Moreover, this scenario (ROS ↔ ABA crosstalk) is consistent with the important role of root ABA in the HS-mediated shoot promoting action (Olaetxea et al., 2015).

3. Main relationships between the HS-mediated effects on plant development and some HS structural features

A number of studies have shown that the ability of HS to affect plant growth and physiology is influenced by their structural features (Rose et al., 2014, and references therein). Thus, in a sedimentary HA with low molecular mass, a high content of carboxyl and hydroxyl groups provided high bioactivity in improving lateral root growth in barley
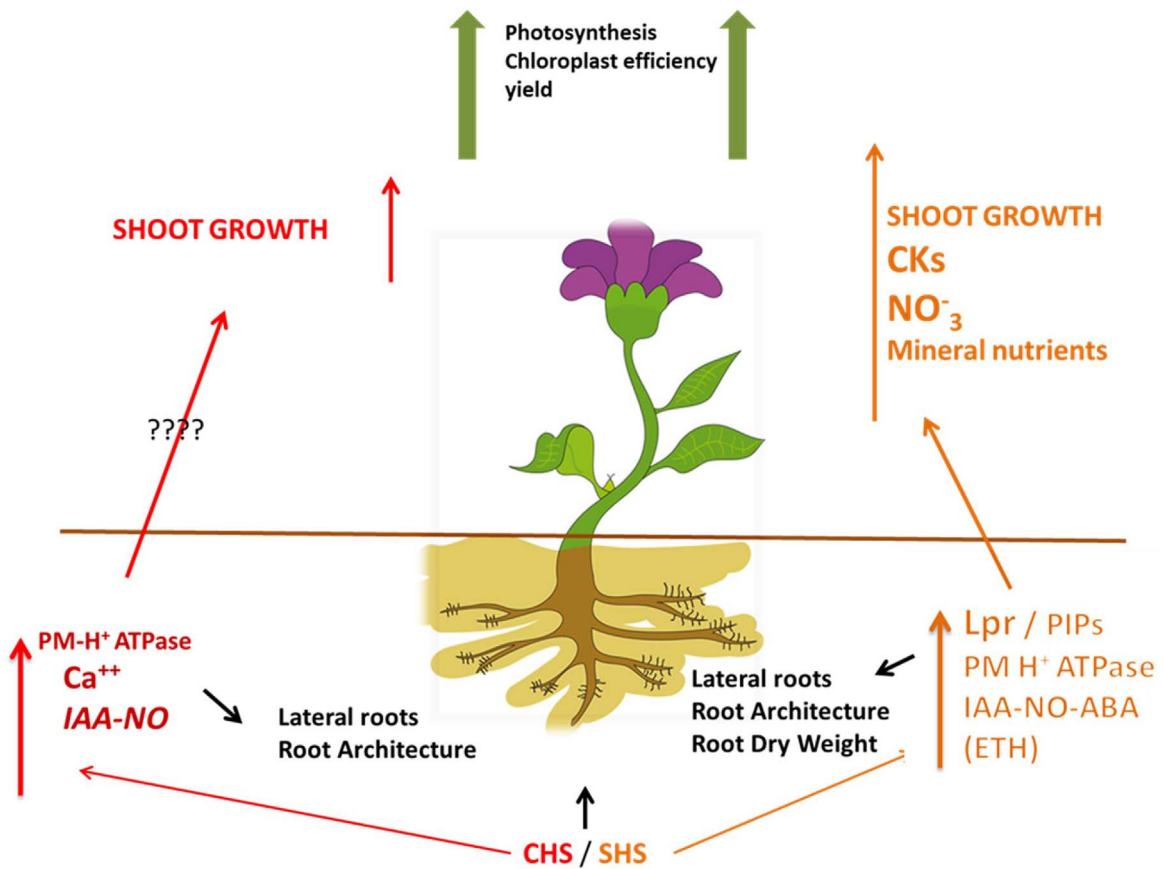
Fig. 9. Root-shoot signalling pathways involved in the shoot growth promoting action of humic substances with diverse origin: CHS (humic substances obtained from compost) and SHS (humic substances obtained from sediments).
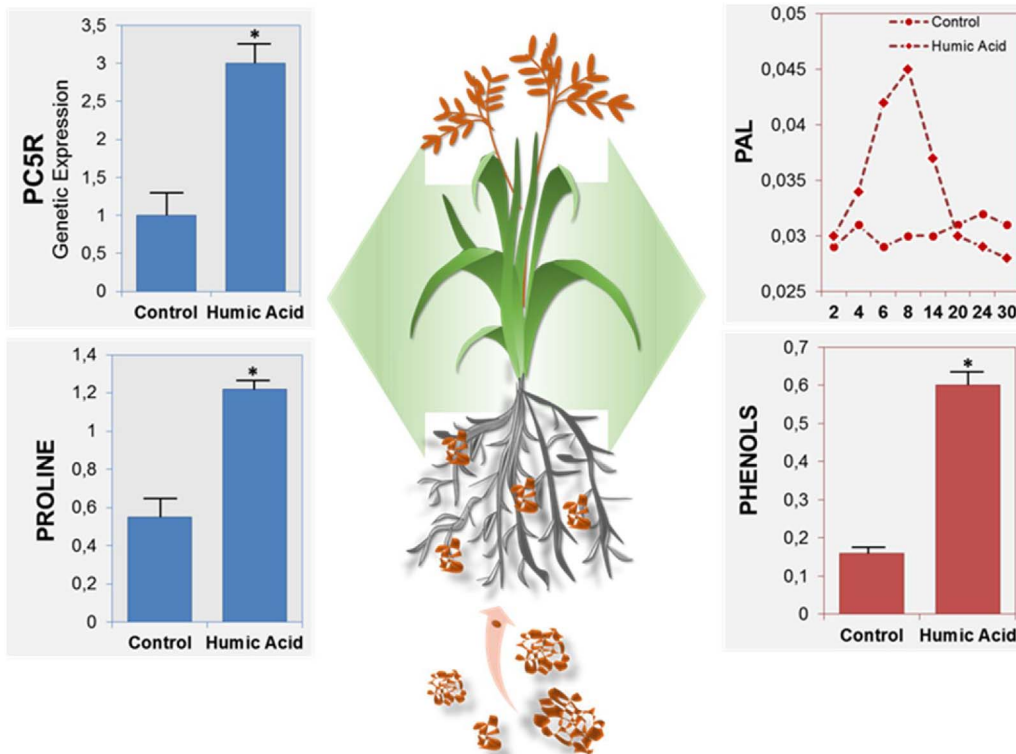

Fig. 10. Effects of humic substances on the signalling pathways involved in the biosynthesis of phenols and proline in rice plants. (PAL: Phenyl-ammonia-lyase;) (from García et al., 2012, 2016aGarcía et al., 2012García et al., 2012, 2016a).
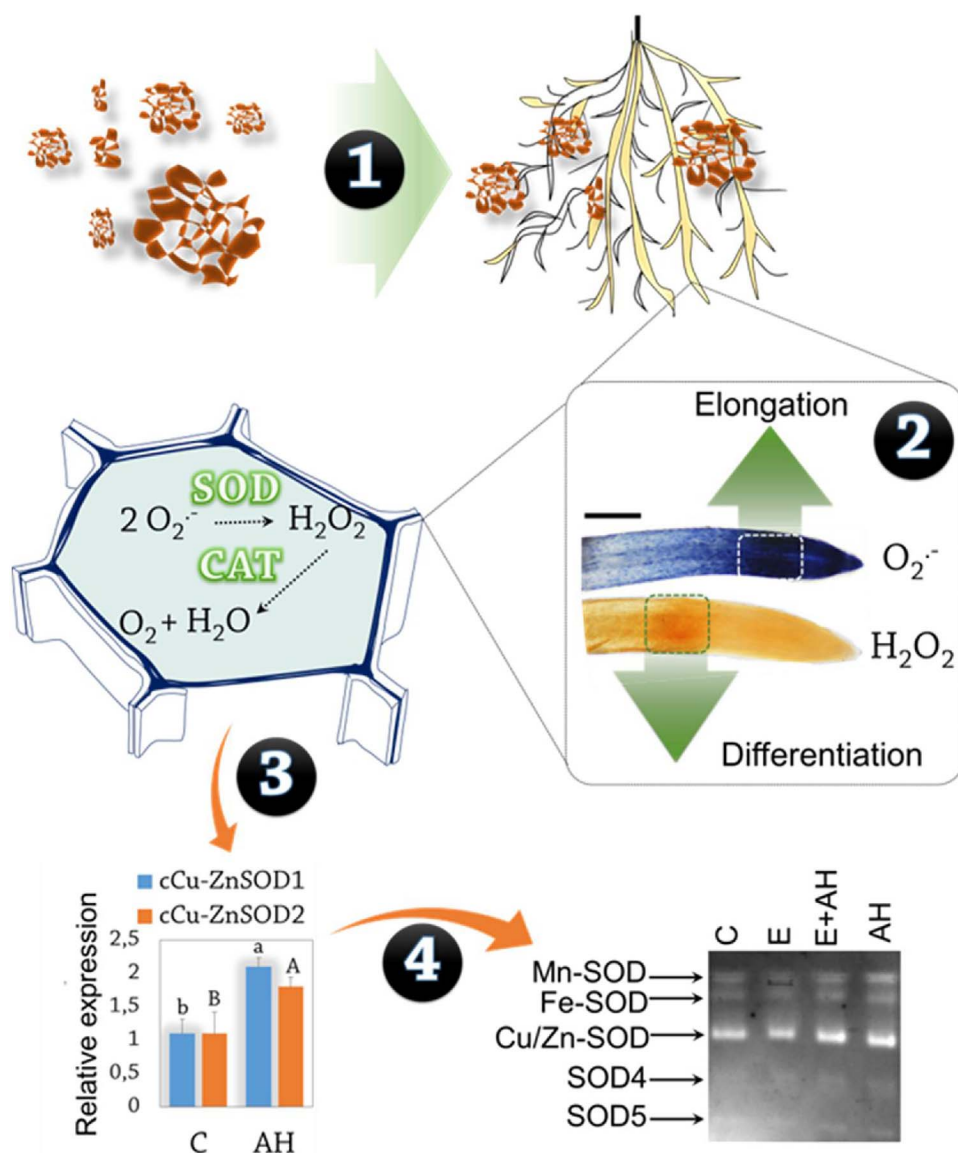
(Piccolo et al., 1992). In addition, less structured and more hydrophilic HS fractions extracted from compost presented a high ability to induce N metabolism enzymes in corn (Vaccaro et al., 2009).

The conformational flexibility of HS may promote higher diffusion of bioactive humic fragments into corn cells and increase the enzyme activity related to glycolysis and the tricarboxylic acid cycle (Nardi et al., 2007). In a study comparing HA fractions with low (< 3500 Da) and high (> 3500 Da) apparent molecular weights, only the fraction with a low content of aromatic structures and a high content of carboxyl groups was able to interact with the cell membranes of carrot plants (Muscolo et al., 2007).

Hydrophobicity and hydrophilicity may also be related to the biological activity of HS. A HA hydrophobicity index presented a strong correlation with the effects of HS on root growth and auxin-like activity in corn, thus indicating that hydrophobic structures with a certain level of lability are probably needed for HS bioactivity (Dobbss et al., 2010). Other studies suggested that polar humic fragments included (and preserved) in hydrophobic domains in the humic structure may be essential for the stimulation of root growth in corn (Canellas et al., 2012). Taken together, these results indicate that HS-mediated root growth promotion seems to be more closely related to HS mobility, molecular conformation and functional group distribution in HS structure than to molecular weight (Canellas et al., 2010).

Studies in corn showed that the ability of HA extracted from vermicompost to promote both lateral root growth and root PM H^+-ATPase activity correlated positively with methoxyl, aryl, O-aryl and carboxyl groups, and correlated negatively with O-alkyl and di-O-alkyl and C-alkyl structures (Aguiar et al., 2013).

Recently, a study including HAs and HSs (HA + FA) extracted from several soil types and vermicompost was able to discriminate the HS structural features related to lateral root promotion from those related to whole root growth promotion in rice (García et al., 2016b). This study included a complete HS structural characterization using ^{13}C NMR, elemental analysis and FTIR, as well as a complete description of root features (number of roots, root length, root diameter, root dry weight and root surface area). The application of complementary statistical tools such as principal component analysis and multivariate curve resolution, allowed the authors to establish the relationships between the main structural descriptors of HSs and HAs and their morphological effects in roots (Fig. 12). Likewise, new structural descriptors such as structural recalcitrance and lability were determined from the multivariate curve resolution data. The results obtained from elemental analyses indicated that while the HS extracted from vermicompost were related to increases in lateral root proliferation, surface area and radicle length, the HS extracted from soil were related to the number of larger roots and root diameter. In this line, the results
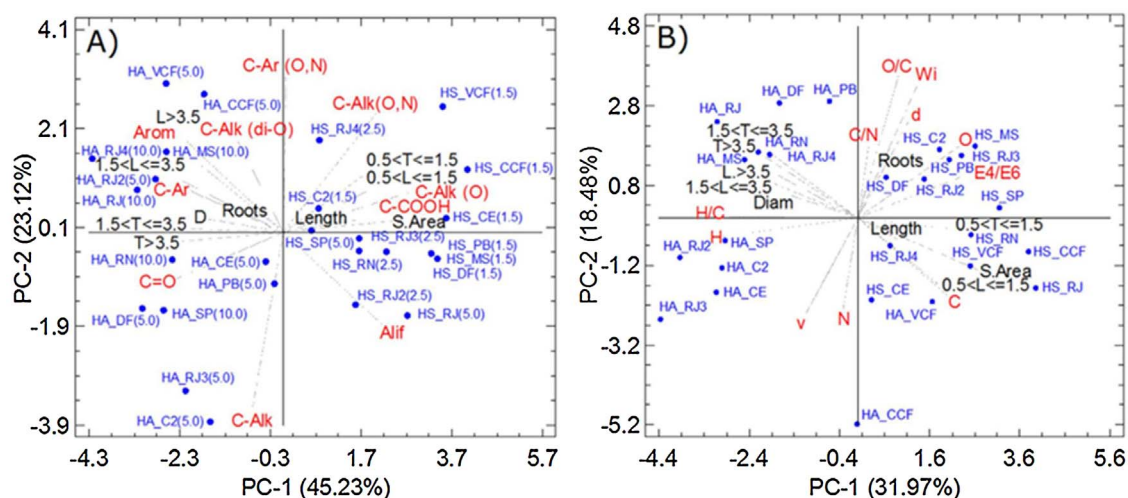
Fig. 12. Relationship between carbon types (13C-CP/MAS-NMR) (A), elemental analysis (B) of different HS and the root parameters in rice plants treatment obtaining through PCA analysis (from Garcia et al., 2016b).

obtained from ^{13}C-CP/MAS-NMR showed that the complete humic systems (HA + FA) extracted from both compost and soil were related to an increase in lateral root proliferation, surface area and radicle length, with this action being associated with substituted C-aliphatic (C-Alk [O,N], C-Alk-O), carboxylic C-COOH groups and aliphaticity. However, HA fractions were related to increases in the number of larger roots, root diameter and root number, with this action being associated with substituted C-aliphatic (C-Alk [di-O]), unsubstituted C-aliphatic (C-Alk), C-aromatic and carbonyl groups (García et al., 2016b).

The structure-property-function relationships described in this work (Garcia et al., 2016b) are in line with the results obtained in studies on the structure-biological activity of HS using fragmentation techniques, such as gas chromatography/mass spectrometry-GC/MS and pyrolysis coupled to gas chromatography/mass spectrometry-Py-GC/MS. Thus, studies in lettuce (*Lactuca sativa*) reported that the auxin-like activity presented by diverse CHA-VC seemed to be positively related to the presence of carboxylic acids and amino acids (Scaglia et al., 2016).

In summary, these results taken together indicate that:

(a) The action of HS promoting lateral root proliferation, root surface and smaller roots seems to correlate positively with more labile O-containing functional groups, principally carboxylic groups, in aromatic and aliphatic structures. As explained above, this specific action of HS seemed to be regulated by the IAA-NO-ethylene signalling network.

(b) However, the action of HS promoting root dry matter production and whole root growth correlated with the presence of more recalcitrant O- and N- alkyl moieties. As discussed above, these latter effects cannot be explained by HS-action on IAA, NO and ethylene in roots, and must involve different signalling pathways dependent on other signals such as ROS for instance.

(c) In consequence, different qualitative HS-mediated effects on root development, which in turn are regulated by different signalling pathways, seem to be related to different structural features of HS.

4. Main events occurring in the HS-root surface interaction

A very relevant question that remains unanswered concerns the nature of the primary event (physical and/or chemical) that occurs when HS interact with the membrane of cells on the root surface. This event (or events) would trigger all the chain of interconnected molecular events that are responsible for the final action of HS-promoting root and shoot growth.

Some authors suggested that the interaction of HS with exudates in

acidified local sites at the root-soil interphase may promote HS disaggregation and further release of phytoregulators (and/or phytoregulator precursors) embedded in inner domains within the structure of HS (Trevisan et al., 2010a; Muscolo et al., 2013; Canellas et al., 2015) (Fig. 13). For HS extracted from composted or vermicomposted organic materials, this hypothesis is very plausible since these materials are quite "young" and probably contain traces of phytohormones. However, for SHS extracted from ancient sediments this hypothesis is unlikely. In fact, many of the studies made with leonardite SHS included an extensive analysis of the main plant hormones related to HS action (IAA, ABA and cytokinins), which yielded negative results (Aguirre et al., 2009; Mora et al., 2010).

The presence in the HS structure of structural domains with steric and electronic similarities to auxins has also been proposed (Trevisan et al., 2010a) (Fig. 13). However, bearing in mind the complexity and size of HS structural units in the HS supramolecule in comparison with auxin molecular sizes and the nature of auxin-receptor interaction (Calderon-Villalobos et al., 2010), this hypothesis appears to be quite improbable.

Recently, some studies have suggested that this primary event resulting from HS-root interaction on HS root application might be physical rather than chemical, and could be associated with a kind of mild stress caused by HS accumulation on the root surface (Asli and Neumann, 2010; Berbara and Garcia, 2014; Olaetxea et al., 2015) (Fig. 13). Some authors have described a type of plant stress associated with the accumulation of inorganic (mainly clays) and organic compounds at the cell membrane on the root surface (Asli and Neumann, 2009, 2010). These authors refer to this type of stress as "colloidal stress". Significant HS accumulation on the root surface has been reported by several studies (Fig. 14) (Garcia et al., 2012). In fact, studies using tritium-labelled HS reported a significant accumulation of HS fractions in root apoplast (Kulikova et al., 2016). In this context, some authors related the primary action of HS in roots to a type of colloidal mild (beneficial) stress associated with a partial HS accumulation at root surface (Berbara and García, 2014; García et al., 2012, 2013, 2016a) (Fig. 13). This hypothesis is consistent with the hypothesis proposed by other authors suggesting that HS root application might cause some type of mild, beneficial, stress as a result of a transient accumulation of some HS fractions on cell walls on the root surface, followed by conformational changes in HS structure and some type of cell wall pore cleaning (Olaetxea et al., 2015). Thus, based on previous studies showing that high concentrations of SHA may induce fouling in cell wall pores on the root surface, thus inhibiting water uptake and causing drought stress and decreasing shoot growth (Asli and Neumann,

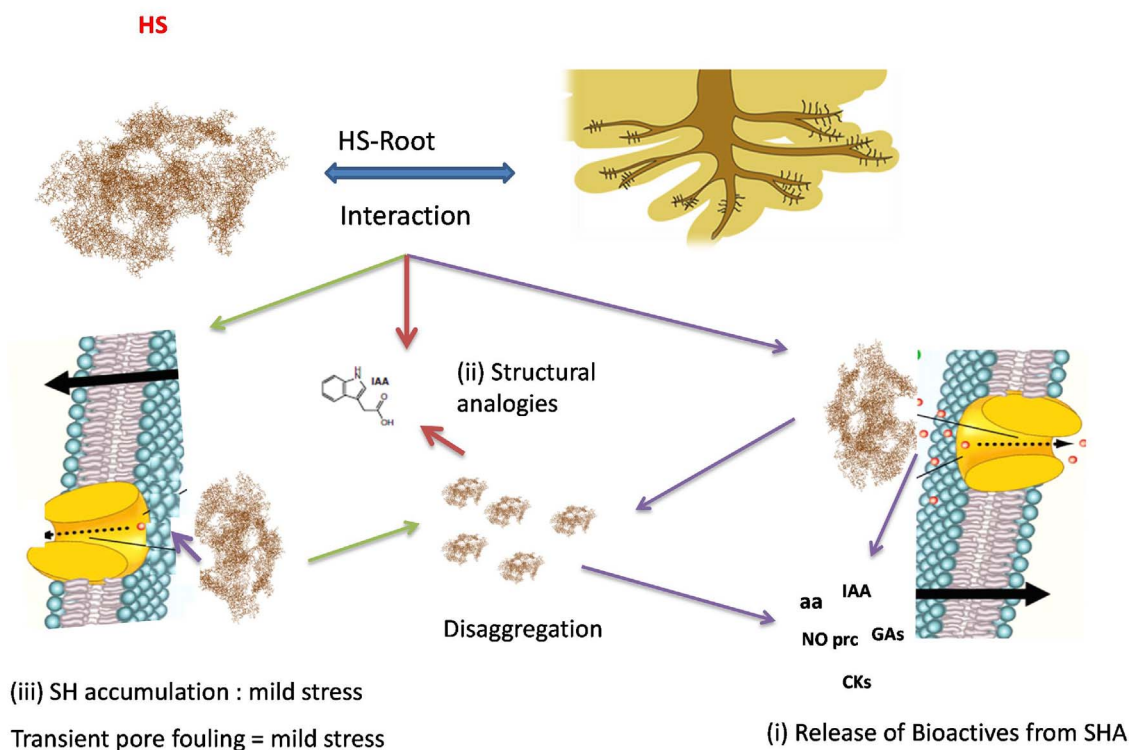## Hypotheses on the primary action of HS at root surface

Fig. 13. Hypotheses for the primary event arising from the interaction of humic substances at root surface: (i) release of phytoregulators upon molecular dissagregation; (ii) auxin structural analogues in the structural humic domains; (iii) mild stress caused by the accumulation of humic fractions at root surface and molecular dissagregation processes.

2010), further studies involving leonardite HA concentrations associated with plant growth promotion have indicated that after a transient stress, probably caused by fouling in root cell wall pores, root hydraulic conductivity and water uptake increased, which is essential for the leonardite HA-mediated enhancement of shoot growth (Olaetxea et al., 2015). The authors suggested that these changes in root hydraulic conductivity might be related to HA disaggregation or some type of conformational change resulting from the action of root exudates and acidic local pH (Olaetxea et al., 2015) (Fig. 13).

In fact, although plant stress effector is defined as any unfavourable condition or substance that affects or blocks plant metabolism, growth or development (Lichtenthaler, 1996; Kranner et al., 2010), some studies have shown that under some conditions, low levels of stress may improve plant metabolic function or response. This is known as "eustress" (beneficial stress), whereas detrimental stress is termed "distress" (Kranner et al., 2010; Petrov et al., 2015). Therefore, in this hypothetical framework, the primary event resulting from HS-root interaction would be a type of mild and transient eustress that might trigger all the downstream chain of events leading to plant growth promotion.

5. Concluding remarks and future potential research

Summarizing, we may underline the following key points:

1. One of the main fractions of NOM that has significant effects on plants and microorganisms is the organic matter dissolved in soils
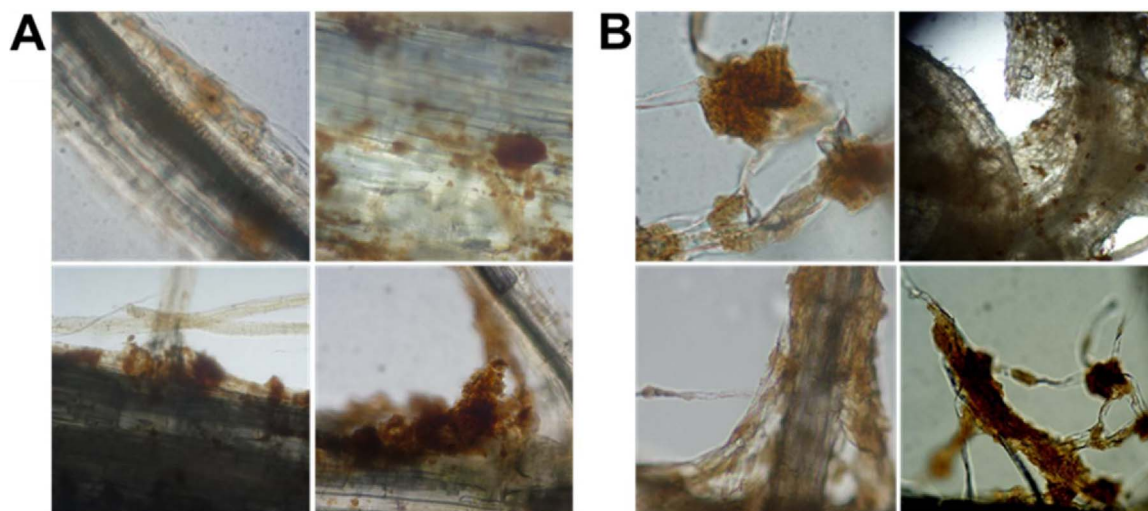

Fig. 14. Interaction of HA with radicular epidermis in rice plant (A) HA agglomeration in the lateral roots (B) (Modified from García et al., 2012).

solutions and aquatic reservoirs (e.g. lakes, rivers), referred to as DOM.

2. DOM in soil solutions or aquatic reservoirs results mainly from the natural solubilisation of specific fractions of NOM and the mineral phase by the action of water in soil, mainly in the A and B horizons.

3. DOM includes components that range from non-degraded organic molecules arising from fresh organic matter (plant and animal debris), such as amino acids, proteins, sugars, polysaccharides, organic acids, metabolites and so on, to molecules resulting from the partial or total degradation of fresh organic matter by the action of microbiota and/or physico-chemical processes (e.g. redox reactions and polymerization). The organic fraction corresponding to the biologically or chemically degraded fresh organic matter is called soil humus.

4. Some of the organic molecules included in DOM form spontaneously in water solution stable molecular aggregates consisting of non-degraded and degraded (transformed) molecules held together by the action of weak attraction forces such as hydrogen bonds, the hydrophobic effect, Van der Walls forces and some types of metal bridges. As a result, DOM presents large heterogeneity and polydispersity. This DOM fraction is known as HS in DOM.

5. HS contain macromolecules as well as simpler molecules, which can be included in molecular aggregates. When molecular aggregation leads to aggregates with new physicochemical and/or biological properties different from those of their individual components, HS may be regarded as a type of natural supramolecule. Some properties of HS seem to emerge from the formation of molecular aggregates in solution and solid phase, such as amphibility and biological recalcitrance. However, specific studies that aim to demonstrate that these properties are not present in the individual components of HS molecular aggregates are needed in order to better elucidate the supramolecular nature of HS.

6. HS improve plant mineral nutrition and growth through both action on soil (or the growing substrate) mainly related to their ability to form stable complexes with metals, and the activation of the molecular and enzyme network involved in nutrient root acquisition. This action is especially relevant for some nutrients such as Fe, N, and P.

7. HS improve root and shoot growth through an inter-connected action on signalling pathways related to auxin-NO and ABA in roots and to cytokinins in the shoot. This action can also involve other molecular signals such as ROS, and secondary messengers such as Ca^{2+}, and is triggered by a primary action arising from the direct interaction of humic substances with cell walls on the root surface.

8. It is hypothesized that the primary action of HS on the root surface might involve two types of effects associated with the disaggregation of DOM humic fraction on the root surface: (i) the release of phytoregulators embedded in DOM structural domains; and/or (ii) a mild, beneficial, stress associated with the accumulation of humic molecules on the root surface and the partial fouling of cell wall pores on the root surface.

9. In intact, natural, ecosystems the main action of HS in DOM on plant growth is probably associated with improvements in micronutrient availability for plants and microorganisms since its concentration in soil solution is very low. However, in anthropogenic ecosystems (mainly agricultural ecosystems), the action of HS in DOM on plant growth also involves direct interaction with plant roots since HS concentration in soil solution and the root area is high.

10. The diverse types of actions of humic substances on plant growth and development are closely related to their chemical structural features, as well as to their molecular conformation in water solution.

Finally, the degree of certitude of some of the molecular and biochemical events induced by the application of HS on plant roots is

Table 1
Description and degree of certitude of some HS-mediated events caused in plant shoot and root and thir relation to plant growth promotion.

HS activity on plant development	Degree of certitude	Experimental conditions	Type and Origin of HS	Role in open field
Metal complexation	A fact	hydroponics, soil	HA and FA from leonardite, peat, soil, compost	A fact whose real relevance has to be studied more in depth for each soil-crop system and experiment
Nutrient root uptake and nutrient transporters in root	A fact	hydroponics	HA from leonardite, peat, compost	hypothetical
Root development: Increase PMH+–ATPase	A fact	hydroponics	HA from leonardite, peat, soil, compost	hypothetical
Root development: Involvement of NO and IAA	A fact	hydroponics	HA from leonardite, compost	hypothetical
Root development: Involvement of Ethylene and ABA	A fact	hydroponics	HA from leonardite	hypothetical
Root development: Involvement of ROS	A fact	hydroponics	HA from compost	hypothetical
Root development: Involvement of Ca^{++} and Protein phosphorilation	A fact	hydroponics	HA from compost	hypothetical
Shoot development: Root to shoot cytokinin translocation	A fact	hydroponics	HA from leonardite	hypothetical
- Primary action on the root: - HS Desaggregation and release of phytoregulators	Hypothetical	hydroponics	HA from soil, compost	hypothetical
- Primary action on the root: - HS Desaggregation and mild stress	Hypothetical	hydroponics	HA from leonardite, peat, compost	hypothetical

summarized in Table 1. In this table we describe the specific effect of HS; the degree of certitude that is defined as a fact when it has been demonstrated experimentally or as hypothetical when it has been proposed but not demonstrated; the origin and type of the HS used in the experiments; and also the degree of certitude and potential relevance of these HS-mediated processes in the effect of HS on plants cultivated in the field. It is noteworthy that the knowledge about the role of many of the processes induced by HS in plants growing in hydroponics in the whole HS action on field crops is scarce. This fact points to the need to develop specific research programs in order to better know the real efficiency of HS in improving field crops yields and quality, the mechanisms responsible for these effects, as well as the influence of environmental conditions, soil type, soil microbiota and plant species. In this sense, future research integrating the interactions of HS on soil microbiota and plant microbiome is of great interest.

Acknowledgements

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