Universidad de Navarra

## Facultad de Ciencias

# Effects of a sedimentary humic acid on plant growth: study of the general mechanisms of action upon root application in cucumber plants (*Cucumis sativus* L. cv Ashley).

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## SUMMARY

HS (humic substances) constitute one of the most abundant forms of organic matter in the biosphere. Many studies reported the ability of humic acids (HAs) isolated from HS to affect plant the plant development of diverse plant species. However, the mechanism of action that is behind these events is scarcely understood and it is not integrated into a holistic view.

To this end, the aim of this work was to study the mechanism of action of an specific sedimentary humic acid (SHA) under roots application in cucumber plants (*Cucumis sativus* L. cv Ashley) in detail. More specifically, the potential role of hydraulic conductivity ($Lp_r$) linked to root abscisic acid (ABA) concentration and some of water relations such us stomatal conductance ($G_s$) and leave ABA concentration in the SHA shoot growth promotion activity. Indeed, the functional relationship between $Lp_r$ with root-ABA biosynthesis and root plasma membrane aquaporins (*Cs*PIPs) molecular regulation was also investigated. With the aim to integrate the studied SHA root mechanism into previously SHA enhanced potential response, the relative role of SHA mediated PM-$H^+$-ATPase activity and $Lp_r$ on shoot and/or root growth promotion of cucumber plants was also assessed. Indeed, the SHA-mediated regulation of root genes associated with root growth was also studied in *Arabidopsis thaliana* seedlings. Finally, with the purpose of studying the relationship between the chemical structure of SHA and the biological activity exerted in plants, a sequential fractionation process named “humeomics” was applied to SHA, and the behavior of some of the obtained fractions was assessed in cucumber plant biological activity.

The results showed that the beneficial effect of SHA on shoot growth was associated with a significant increase of $Lp_r$ and $G_s$. The SHA mediated increase in Lpr was root-ABA dependent and regulated by some of the root aquaporin homologues. Regarding the discriminating role of SHA enhancing $Lp_r$ activation or PM-$H^+$-ATPase activity in both shoot and/or root growth, results showed that the SHA-mediated enhancement of root PM-$H^+$-ATPase activity might play a secondary role in the improvement of shoot growth caused by SHA, $Lp_r$ activity being more relevant. Furthermore, our results indicated that probably the stimulation of root PM-$H^+$-ATPase activity plays a more

relevant role than the increase of $Lp_r$ in the mechanism of action of SHA on plant root growth.

In relation to the structure-activity relationship using humeomic fractionation approach in cucumber, results showed that 62.5% of the extracted material corresponded to the unbound fraction (ORG 1). In the determination of biological activity, two fractions were studied: the mentioned ORG 1 fraction and the remained residue (RES 1). The main structural difference between both samples was that RES 1 fraction showed higher aromatic character than ORG 1, which was more aliphatic. RES 1 fraction increased $Lp_r$, root and shoot growth compared to control and bulk SHA treated plants. ORG 1 fraction, instead, did not show significant differences on $Lp_r$ or shoot growth compared to control or SHA treated plants. The response in root growth was similar to SHA bulk treatment.

As for the SHA regulated root gene expression in *Arabidopsis thaliana*, results showed that there were several up-regulated genes encoding root promotion ability functions, such us AT3G15370.1 gene function, identified as a member of an Alpha-Expansin Gene Family or AT2G14960.1 gene that encodes a protein similar to IAA-amino synthetase, which maintains the auxin homeostasis. Moreover, some other up-regulated genes that could be related with SHA responses that other authors previously reported were also identified.

## RESUMEN

Las sustancias húmicas (SH) constituyen una de las formas más abundantes de materia orgánica de la biosfera. Muchos estudios anteriormente publicados describen que los ácidos húmicos (AH) extraídos previamente de las SH poseen la capacidad de afectar al desarrollo de plantas de diversas especies. Sin embargo, la comprensión del mecanismo de acción que subyace a este efecto es escasa y no está integrada en una visión holística sobre el tema.

Con este fin, el objetivo principal de este trabajo fue estudiar en detalle el mecanismo de acción de un ácido húmico específico de origen sedimentario (AHS) aplicado radicularmente en plantas de pepino (*Cucumis sativus* L. cv Ashley). Concretamente se estudió la participación potencial de la conductividad hidráulica ($Lp_r$) unido a la síntesis de ácido abscísico (ABA) en raíz junto a otras relaciones hídricas tales como la conductancia estomática ($G_s$) o la síntesis de ABA en hoja sobre la actividad promotora del AHS en el desarrollo vegetal de la parte aérea. Además, se investigó la relación funcional entre $Lp_r$ y ABA en raíz con la regulación molecular de las acuaporinas de la membrana plasmática en raíz (*Cs*PIPs). Con la intención de integrar el citado mecanismo radicular del AHS en respuestas activadas por AHS y descritas anteriormente, se estudió la implicación relativa de la activación de la ATPasa de la membrana plasmática (PM-$H^+$-ATPase) y de la $Lp_r$ sobre la estimulación del desarrollo de la raíz y parte aérea en plantas de pepino. También se estudió la regulación de los genes de la raíz por acción del AHS, en plántulas de *Arabidopsis thaliana*. Finalmente, con el objetivo de estudiar la relación estructura química de AHS- actividad biológica ejercida en planta, se llevó a cabo un fraccionamiento secuencial del AHS denominado "humeómica" y se estudió la actividad biológica derivada de la aplicación de ciertas fracciones en plantas de pepino.

Los resultados mostraron que el efecto beneficioso mostrado por AHS en el crecimiento de la parte aérea estaba unido a un incremento significativo de $Lp_r$ y $G_s$. Este aumento de $Lp_r$ mostró ser dependiente de ABA-radicular y estar regulado por ciertos hómologos de las acuaporinas de la membrana plasmática de células radiculares. En relación al estudio de la relevancia ejercida por AHS en la activación de PM-$H^+$-ATPase o $Lp_r$ como respuesta al crecimiento de la raíz y/o parte aérea, los

resultados mostraron que la activación de la PM-$H^+$-ATPase mediada por AHS jugaría un papel secundario en el beneficio del desarrollo de la parte aérea, siendo más relevante la activación de $Lp_r$. Además, nuestros resultados también indicaron que probablemente la estimulación de PM-$H^+$-ATPase sería un factor más relevante que el incremento de $Lp_r$ sobre el crecimiento de la raíz.

En relación al estudio de estructura-actividad biológica realizada aplicando el fraccionamiento "humeómico", los resultados mostraron que el 62.5% del material extraído correspondía a la fracción no enlazada (ORG 1). Para la determinación de la actividad biológica, se estudiaron dos fracciones: la mencionada fracción ORG 1, y el remanente del fraccionamiento (RES 1). La principal diferencia estructural entre las dos fracciones fue que la fracción RES 1 presentaba un carácter más aromático que la fracción ORG 1, cuyo carácter fue más alifático. La aplicación de la fracción RES 1 en planta incrementó $Lp_r$, el crecimiento de la raíz y parte aérea en comparación al control y al AHS integral. Sin embargo, la fracción ORG 1, no presentó diferencias significativas en $Lp_r$ o crecimiento de la parte aérea en comparación al control o al AHS integral. La respuesta de la aplicación de ORG 1 en el crecimiento radicular fue similar al mostrado por el AHS integral.

Con respecto a la regulación de genes radiculares ejercida por la aplicación de AHS en *Arabidopsis thaliana*, los resultados mostraron que entre los genes sobre-expresados, algunos codificaban para respuestas relacionadas con la estimulación del desarrollo radicular, como el gen AT3G15370.1, identificado como un miembro de la familia de los genes de Alpha-Expansinas, o el gen AT2G14960.1, que codifica para una proteína similar a IAA-amino synthetase, el cual mantiene la homeostasis de auxinas. Además, también fueron identificados otros genes sobre-expresados que relacionaban respuestas previamente descritas por AHS en planta por otros autores.

# INTRODUCTION

Ever since human beings have started working in agriculture, the native soil organic matter (SOM)* has been used as a plant growth promoter. From the beginning of the modern era, the relationship between soil fertility and crop yields with the presence of a specific fraction of soil organic matter known as humus was observed and extensively studied (Macarthy et al. 1990; Chen et al. 2004). Many studies also reported the beneficial action of soil humus on the growth and mineral nutrition of different crop species (Lee et al., 1976; Canellas et al. 2002; Clapp et al. 2001; Chen et al. 2004).

From a more chemical standpoint, the general concept of humus is commonly called humic matter or humic substances (HS). Although the definitions of the term changed depending on the point of view of each of the researchers, humic substances proposed by Stevenson (1994) can be defined as: a series of relatively high-molecular weight, brown to black colored substances formed by secondary synthesis reactions. This term is used as a generic name to describe the colored material or its fractions obtained on the basis of solubility characteristics. These materials are distinctive to the soil (or sediment) environment in that they are dissimilar to the biopolymers of microorganisms and higher plants (including lignin).

MacCarthy and Suffet (1989) defined the term *humus* or *humic substances* as the organic material in the environment that results from the decomposition of plant and animal residues, but that does not fall into any of the discrete classes of compounds such as proteins, polysaccharides, polynucleotides, and so on.

Humic substances are naturally produced in soil by the chemical and/or biochemical transformation of fresh organic matter coming from plants and/or animals. This transformation-degradation process is called humification process and it can evolve for a long time, even thousands of years. From a biochemical perspective, these reactions involve soil microbiota and soil enzymes. With regard to the physico-chemical environmental conditions, oxygen availability, soil water content, soil temperature, soil pH, texture, etc. are involved in the process (Stevenson 1994; Hayes 2009; Huang and Hardie 2009). Biochemical or physico-chemical and fresh organic matter origins condition the humification process and its result.

Considering the evolution of organic farming in the XIX century, the composting process is a technique in which the chemical and biological transformations of fresh organic matter are induced under aerobic conditions. As a result, this accelerated humification process produces partially humified HS (Haug 1993).

Among the different theories trying to explain the nature of the humification process, the most generally accepted theory is that humic substances are derived from lignin. Since lignin is one of the three major components of wood, it could be degraded by soil microorganism, thus becoming part of the soil humus (Stevenson 1994). The above mentioned general idea of humification process involving lignin is, in turn, explained by three specific theories: the ligno-protein theory, the phenol-protein theory and the sugar-amine condensation theory.

The lingo-protein theory:

This theory was first developed by Waskman (Waskman 1932) although it was Stevenson (Stevenson 1994) who renamed it as the *lignin-degradation model*. Briefly, lignin would be degraded by microorganism action, generating hydroxyphenols and oxidizing the aliphatic chains until formation of corresponding carboxylic acids. These last mentioned functional groups would react with amino groups, thus generating humins as a first reaction (because humins are the most complex humic substances). These humins would gradually be degraded, forming the corresponding humic acids and fulvic acids.

Phenol-protein theory:

This theory was also renamed by Stevenson (1994) as *non-lignin-polyphenol-polymerization model* in which the decomposition of cellulose forms polyphenols that enzymatically oxidize to quinones and then, via polymerization, would generate humic substances.

Sugar-amine condensation theory:

This theory is also known as *the melanoidin pathway* (Nissenbaum 1972; Hatcher et al., 1985) or the Maillard reaction theory, as suggested by Stevenson (1994) and Ziechmann (1994). In this proposed theory, sugars and amino groups would react by

nonenzymatic or abiotic pathways, producing melanoidins which by final polymerization reaction would produce different humic substances.

Once the general definition of HS and the main benefit on farming have been stated, the introduction delves deeper into the description of HS, showing the state of art of the principal actions of HS on plants.

**Main classification of humic substances**

As for the classification of HS, water solubility, as a function of pH, is considered the main physico-chemical feature to organize HS-s. There are three main fractions coexisting in HS (Stevenson 1994):

- Humic acids (HA), the fraction of HS soluble at alkaline pH (in water solution) but insoluble at acid pH
- Fulvic acids (FA), the fraction that is soluble at all pHs (in water solution)
- Humin, insoluble at all pH values (in water solution)

According to this definition, there is an HS extraction methodology that was created in 1981 by a group of scientists. It was included in the International Humic Substances Society (IHSS) in order to develop a common extraction method and make the definition step of HS easier (International Humic Substances Society, 2015). This methodology is based on a first alkaline extraction with aqueous NaOH, followed by a precipitation step at low pH in which humic acids precipitate, and some final desalting steps (cation exchange, dialysis…) of the solution where fulvic acids coexist.

With the study of humic substances, this methodology has been temporary updated in terms of the latest knowledge agreements. However, this operational methodology addressing a general physico-chemical feature of HS makes obtaining humic fractions

* When it refers to the soluble fraction of SOM (water soluble and/or alkali soluble), this fraction is usually called dissolved organic matter (DOM) (Stevenson 1994). By analogy, when studies are carried out in hydroponics or inert substrates cultivation media, the use of the terminology of DOM is generally accepted.

from any type of natural or modified organic matter (wood, paper, textiles, polymerized materials…..) possible, but at the same time, this perspective rejects the real humification degree of humic matter. There is another useful classification of humic substances principally addressing this humification degree (Mora et al. 2014b).

- Artificial HS: organic substances extracted by IHSS-method from transformed or modified organic materials by using an alternative or complementary process different from natural composting. Controlled pyrolysis can be used to obtain biochar (Schulz et al., 2013), carbohydrate-derived fulvates (Sherry et al., 2013), phenol-derived humates (Fuentes et al. 2013), etc. These HS are called Artificial HS (AHS) and their fractions are called artificial humic acids (AHA) and artificial fulvic acids (AFA).
- Fresh HS: organic substances extracted by IHSS-method from neither biologically nor chemically modified fresh (living) organic materials, such as plant or animal fresh residues (leaves, whole plant root or shoot, wood, weeds…). These HS are named fresh HS (FHS) and their fractions are called: Fresh humic acids (FHA) and fresh fulvic acids (FFA).
- Compost HS: organic substances extracted by IHSS- method from composted organic materials (Haug, 1993). These HS are named compost HS (CHS) and their fractions are as follows: compost humic acids (CHA) and compost fulvic acids (CFA).
- Sedimentary HS: organic substances extracted by IHSS-method from naturally humified organic matter with sedimentary origin present in terrestrial (soils, coal, leonardite, peats) and aquatic (lakes, rivers, sea) environments (Stevenson, 1994). These HS are called Sedimentary HS (SHS) and their fractions are referred to as sedimentary humic acids (SHA) and sedimentary fulvic acids (SFA).

The nature of humic matter used to develop all the experiments was sedimentary. Specifically, these SHS were extracted from leonardite (Danube basin) and after the extraction and purification method based on IHSS, SHA fraction was used in the study.

Among all the HS classified above, only SHS and CHS should be considered as being real HS, in terms of both definitions presented.

**Molecular view of humic substances**

It is a fact that the study of the molecular structure and conformational features of humic substances has been and continues to be a big challenge for researchers.

There are two general views regarding the behavior of HS in solution:

The classical view describes HS as natural polyelectrolytes with polymer-based structure forming polydisperse and heterogeneous molecular systems with a major molecular behavior in solution, depending on the elemental composition of the molecules, ionic strength and pH of the solution (Swift 1989; Clapp and Hayes 1999).

On the other hand, the modern view considers HS as being relatively simple molecules held together through weak (non-covalent) binding forces, such as hydrogen bonds, hydrophobic or van der Waals forces, forming a supramolecular entity (Piccolo 2002). From this perspective, some studies also described the micelle character and surfactant properties of HS in solution (Clapp and Hayes 1999, Wershaw 1999).

Contrary to these isolated point of views, Baigorri et al. 2007 (Baigorri et al., 2007b) recently described that both behaviors coexist simultaneously in humic systems. This theory describes the relevance of each of the behaviors as a function of HS solubility, depending on pH and ionic strength defined for the solution of study. Thus, humic acids that are insoluble at alkaline-neutral pH and high ionic strength present macromolecular behavior in solution, whereas humic acids that are soluble at both alkaline-neutral pH and high ionic strength shared both macromolecular and supramolecular behaviors. Finally, fulvic acids, that are soluble at all pHs have supramolecular behavior (Baigorri et al. 2007 a, b).

These conclusions regarding the conformational behavior presented above and some more conclusions regarding the characterization of HS have been reached thanks to several analytical techniques: NMR spectroscopy (Simpson et al., 2001), high-performance size exclusion chromatography (HPSEC) (Piccolo et al., 2002), dynamic light scattering (DLS) (Baigorri et al. 2007a), pyrolysis-gas chromatography/mass

spectrometry (GC/MS) (Martin et al., 1995; Lehtonen et al., 2000), UV and fluorescence spectroscopy (Fuentes et al., 2006), among others.

In general, the use of an analytical technique is normally coupled with a first extraction or physico-chemical optimization procedure, such as the general extraction procedure proposed by IHSS coupled with UV or fluorescence, the ultrafiltration procedure coupled with DLS measurement or pyrolysis coupled with chromatography/mass spectrometry (GC-MS) etc. Therefore, the use of these types of initial procedures for elucidating the structure of the molecules coexisting in humic substances is unavoidable, although sometimes the chemical structure of the natural humic substance can be partially modified.

Along these lines, a new fractionation method has recently been proposed (Nebiosso and Piccolo 2011). This new fractionation method suggests that the humic supramolecular original structure can be reduced by progressively breaking inter- and intramolecular interactions between the humic entities. Consequently, after this fractionation method is performed, the structural identification of humic structures could be carried out by analytical techniques (Nebbioso and Piccolo, 2011).

However, as MacCarthy and Rice (1991) have reported, although in the history of biochemistry many methods of isolation, fractionation and purification of proteins, polysaccharides, polynucleotides and other biopolymers have been developed, the fundamental quest for structural backbone for humus has not reached the same results as in other matters. The main reason can be attributed to the irregularity and polydispersity that humic molecules present. Although much is known about the functional groups content, acidity, aliphatic or aromatic character of humic substances, it has not been possible to integrate this knowledge within the unified framework of a unique molecular structure or class of structures.

**Ecological and holistic view of humic substances**

The lack of reaching a unified framework regarding the chemical structure of humic molecules could be explained by considering and accepting the very essence of humus and its role in nature as reported by MacCarthy and Rice (1991). In fact, the molecular complexity and irregularity of humus constitutes its elemental character. The

molecular disorder, rather than order, provides the key to an important process in nature. Humus is a prevalent material that can be found in all terrestrial and aquatic environments, constituting one of the most abundant forms of organic matter in the biosphere. Swaby and Ladd (1962) suggested that because of the disordered nature of humus molecules, no enzyme would be capable of rapidly degrading this material and this specific feature will guarantee its persistence in nature. In fact, most biopolymers such as proteins, polysaccharides or polynucleotides undergo relatively rapid decomposition in the soil environments. This decomposition is facilitated by the orderly sequence of monomer units. Taking into account that organisms can evolve until they are capable of degrading or decomposing substrates with regular or polymeric structure, the lack of an ordered and homogeneous nature of humic substances guarantees their persistence in nature providing the ability of supplying the necessary characteristic to soil and plants in the environment for prolonged periods. Although humus is resistant to decomposition, it is not totally recalcitrant and it decays slowly. Microbial degradation is totally necessary for humus formation but this is an equilibrium that needs to be reached in order to guarantee its persistence. In fact, the heterogeneous nature of humus represents the key of its persistence in the environment.

**Beneficial effects of HS on plant development and mineral nutrition. A general view regarding the primary event**

It is a generally accepted fact that the application of HS to plants either cultivated in soils or in other soilless cultivation media such as, perlite, vermiculite or hydroponics affects the growth of both shoot and root as well as plant mineral nutrition. The changes derived by this application generally have beneficial effects on crop farming by influencing plant physiology (Nardi et al., 2000, 2002; Azcona et al., 2011; Canellas and Olivares 2014)

These aforementioned changes on plant physiology are produced by two kinds of effects, influencing the plants directly and/or indirectly influencing their environment.

*Indirect plant environment effects:*

These indirect effects include HS physical action on growth medium (changes in porosity, aggregation, gas exchange), specific chemical actions mainly related with nutrient bioavailability thanks to their inherent ability to form complexes with metals (phosphorus, iron, copper, zinc or manganese) (Stevenson 1994) and biological features related to microbiota activity (Chen and Aviad 1990; Chen et al., 2004).

*Direct effects on plant physiology:*

These direct effects involve local-effects of HS at root and/or shoot plant cell membranes that consequently produce molecular and biochemical changes at transcriptional and post-transcriptional levels within the plant. Some studies show that these direct effects are due to the uptake of a small fraction of HS (MW <3500 Da) into the apoplast area (Vaughan and Malcom 1985; Nardi et al., 2002, 2009), whereas some other authors state that these direct effects are caused by a type of unspecific interaction of HS at cell surface (Canellas et al. 2002; Quaggiotti et al. 2004; Muscolo et al. 2007; Aguirre et al. 2009; Trevisan et al. 2010a).

In fact, the aforementioned first HS-root surface event that causes all these biochemical and metabolic effects within the plant remains under discussion. Mainly because there are two views that try to explain this action. The first point of view considers that the action of HS on plant root, and thereby on the whole plant, is mediated by the possible presence of significant concentrations of plant hormones in HS systems (Nardi et al., 2002; Canellas et al., 2008; Zandonadi et al., 2013) or in some cases because of the presence of molecules with an atomic-functional group configuration similar to certain plant hormones, mainly auxins, that could trigger the same effect as the real plant hormone (Canellas et al., 2011; Trevisan et al., 2010a,b). On the other hand, some studies carried out by other authors show that the application of HS (with sedimentary origin) without the presence of detectable concentrations of plant hormones (IAA, Cks and ABA) in their structure can also trigger these molecular and biochemical responses (Mora et al. 2010; 2012; 2014b; Olaetxea et al. 2015).

Considering that all the studies supporting different point of views are carried out with HS of different origins (as well as with different extraction procedures and different fractions), it is quite difficult to conclude which is the main mechanism that triggers all these metabolic-biochemical effects. Conversely, it could be possible that each of the mentioned HS acts in its own way or in more than one way because the principal HS structural domains and conformational behaviors are different.

Before closing this section, it is important to note that HS action on plant growth depends on some general features that need to be taken into account, such as, characteristics of the substrate, the crop type and its management, the environmental conditions where HS are applied, the plant species and its physiology (Tan and Zow 2001), the type and moment of application and finally, the physico-chemical features of HS which condition their behavior.

**Beneficial effects of HS on plant root growth**

Considering the transcriptional regulation of gene-networks, some studies claim that the application of HS affects certain transcription factors involved in the root uptake and subsequent metabolism of different nutrients, showing positive effects on plant metabolism and plant root development (Aguirre et al., 2009; Trevisan et al., 2010a,b; Azcona et al., 2011). These changes involve up-regulation of transporter proteins of Fe (Aguirre et al., 2009; Pinton et al., 1999), nitrogen-nitrate (Pinton et al. 1999; Quaggiotti et al. 2004; Mora et al. 2010) or sulfur (Jannin et al., 2012), among others. In addition, some studies report the up-regulation of auxin-regulated genes (Trevisan 2009), genes codifying $H^+$- ATPase proteins synthesis (Quaggiotti et al. 2004) and root Fe (III)-chelate reductase activity (Aguirre et al. 2009). Along these lines, some studies also show that the above-mentioned transcriptional-mediated effects were associated with complementary effects on root and shoot concentration of phytoregulators (Mora et al., 2010).

Among the different endogenous plant phytoregulator concentrations that can be modified in root because of the action of HS, there is a relevant relationship between auxins and root features or biological activities, supported by numerous studies (Quaggiotty et al., 2004; Muscolo et al., 2007 Zandonadi et al., 2007; Canellas et al.,

2011; Trevisan et al., 2010b). The main effects caused by CHS through IAA are related to root morphology and to lateral root proliferation (Canellas et al., 2002; Zandonadi et al., 2007; Mora et al., 2010; Trevisan et al., 2010b; Muscolo et al., 2013). In fact, these authors link this effect to the ability of HS to increase root plasma membrane $H^+$-ATPase activity. This root morphology change and growth promotion could derive from IAA-mediated increase of root $H^+$-ATPase activity, in line with the acid growth theory (Hager 2003; Rayle and Cleland 1992).

Apart from the well-known influence of HS on IAA synthesis, more recent studies show that this increment in $H^+$-ATPase activity could be triggered by the root production of NO, consequently involving IAA and ethylene-dependent or sometimes non-dependent pathways (Zandonadi et al., 2010; Mora et al., 2009; 2012; 2014a).

Interestingly, these last studies also show that SHA application causes an increase in ABA-root concentration that is IAA- and ethylene-dependent (Mora et al. 2014a). With regard to the crosstalk between NO and ABA production, this relationship is not clear mainly because inhibition of NO causes an increase in ABA production (in control plants), suggesting that both phytoregulators could possibly trigger parallel hormonal cascades independently.

Recently, complementary studies describing plant lateral root emergence in rice roots were carried out (Ramos et al., 2015). Vermicompost origin humic acid application exerted a positive effect on plant lateral root development by activating $H^+$ and $Ca^{+2}$ fluxed into the root and concomitantly activating $H^+$-ATPase activity and $Ca^{+2}$-dependent protein kinase (CDPK). From a genetic standpoint, these effects were also coupled with two stress responsive CDPK isoforms, OsCPK7 and OsCPK17, and with the activated expression of the voltage-dependent OsTPC1 $Ca^{2+}$ channels. Thus, apart from the known effect of HA on $H^+$-ATPase activity for causing cell emergency or elongation, more metabolic domains are also affected by HA, such as $Ca^{+2}$-dependent protein kinase. CDPKs are enzymes that perceive intracellular changes in $Ca^{+2}$ concentrations and translate these stimuli into specific phosphorylation events to initiate signaling processes further downstream. In fact, several studies suggest that CDPKs act as signaling hubs in plant stress signaling and development (Schulz et al., 2013),

suggesting that the interaction of HA with root triggers complex biochemical and genetic networks that work together, possibly to develop some general effects in plants that are principally related to plant development or protection against stresses or injuries.

Along this line of thought, García et al., (2012) reported another study regarding HA involvement in antioxidative metabolism in rice plants *Oryza sativa* L. This study involved vermicompost HA interaction with rice roots activated enzymes of plant antioxidative metabolism, such as POX (peroxidase), APOX (ascorbate peroxidase), CAT (catalase) and SOD (superoxide dismutase). Concomitantly, the activation of these enzymes controlled the balance of ROS species (principally $H_2O_2$) in plant cells, changing the gene expression of rice tonoplast intrinsic proteins (*Os*TIPs) that act as gatekeepers between the cytoplasm and the vacuolar components of the cell.

**Beneficial effects of HS on plant shoot growth**

It is important to note that while the HS mediated effects on root are extensively studied, shoot promoting HS mechanisms are poorly studied (Mora et al., 2010)

With regard to the post-transcriptional effects, Mora et al. (2010) described the relationship between sedimentary origin humic acid (SHA) root application on root PM $H^+$-ATPase activity and nitrate root uptake with Cks root-to-shoot translocation in cucumber plants. These effects were also linked to improvements in the root-to-shoot translocation of main nutrients.

From a genetic standpoint, further studies carried out in rapeseed showed that SHA application in roots did indeed affect the expression of genes involving CK expression pathways in shoot. Furthermore, gene expressions encoding chloroplast functionality and delaying senescence were also activated (Jannin et al. 2012). This study also described significant improvement of net photosynthetic rates on rapeseed plant which, taken together with previously presented data, suggests that shoot growth promotion effect is a result of all these facts.

As for the complex hormonal network that could explain the SHA exerted response in shoot growth, the results reported by Mora et al., (2014a) showed that shoot growth

promoted by SHA is due to an increase of IAA concentration in the root through both a NO-dependent and NO-independent pathway. In addition, the increased ethylene production in the root is regulated by an IAA-dependent pathway. Finally, results also showed that the increase of ABA concentration in the root is regulated through both IAA and ethylene-dpendent pathway.

Even though the effects of HS are scarcely studied in shoot, new frontiers were recently opened with data published by Asli and Neumann (Asli and Neumann, 2010). Thus, a new mechanism by which HS can adversely affect plant shoot growth was reported. Different sources of organic matter were assayed in maize plants (*Zea mays* L.), such as an unidentified source of coal purchased from Sigma Aldrich, Pahokee peat-derived HAs and leonardite-derived HA, all of them applied in a concentration of 1 g $L^{-1}$ aqueous solution. Humic acid exerted a novel mechanism by which shoot growth, transpiration, resistance to water stress and hydraulic conductivity ($Lp_r$) were reduced. However, root growth was not reduced. It should be pointed out that the concentration of HA used in these assays was much higher than the normally applied HA concentration for agronomic benefits (50-250 mg $L^{-1}$) (Rose et al. 2014). Nevertheless, this new scenario opens novel effects that might be involved in the primary mechanism of action of humic acid, a new physical action that totally depends on HA size, and that affects root permeability and water uptake by the interaction of HA on root surface cells.

**Main prospects of study**

It is of great importance to mention that all the experiments developed in cucumber plants (*Cucumis sativus* L. cv Ashley) are carried out using a sedimentary origin humic acid (SHA) and applied in a concentration of 100 mg $L^{-1}$ aqueous solution.

Among the different aforementioned open fronts, some of them make up the framework on which the following described results are based. One of the main events on which the proposed investigation is focused is to study the mechanism of plant development. For the SHA shoot growth promotion study, it is important to consider the previous shoot growth-related NO-IAA-ABA root signaling pathway dependency (Mora et al., 2013, 2014), as well as the recently described shoot growth involved-HA

ability to modify root hydraulic conductivity (Asli and Neumann, 2010). In relation to root growth promotion, the IAA-dependent action of HS on root plasma membrane $H^+$-ATPase activity would be considered as the main issue for complementing the study (Canellas et al., 2002; Zandonadi et al., 2007; Mora et al., 2010; Trevisan et al., 2010b; Muscolo et al., 2013). Following along this line, we have investigated the effects of SHA at transcriptional level in Arabidopsis root using RNA seq approach.

In order to unify the beneficial action of HA on plant development with the molecular view of HA, some experiments will be carried out along this line. First of all, with the aim of studying the chemical and conformation features of the SHA, the recently described humeomic-based fractionation method associated with some of the HA characterization techniques will be considered. Finally, a relationship between the SHA molecular view and SHA exerted biological activity will be studied.

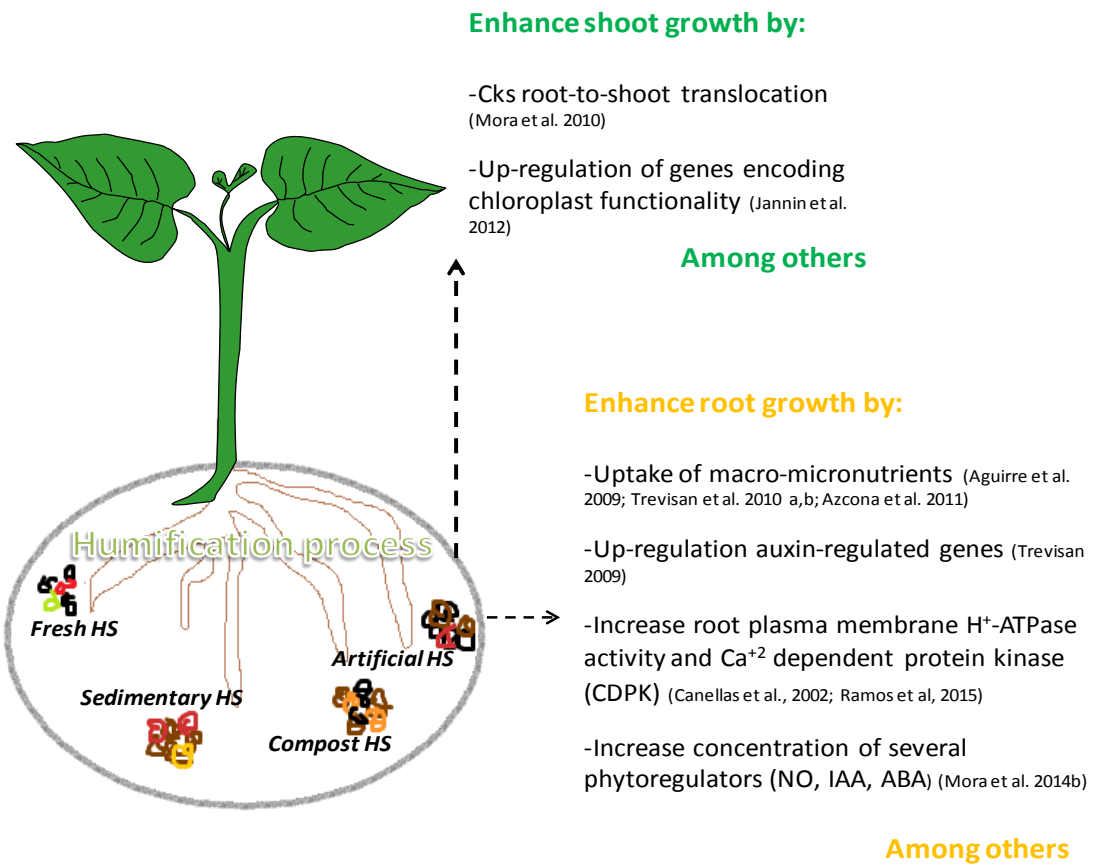


Figure 1: Integrated effects of different origin HS on plant development

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## HYPOTHESIS AND AIMS

1. To study the potential role of hydraulic conductivity in the main mechanism by which SHA promote shoot growth in cucumber (*Cucumis sativus* L. cv Ashley). To investigate the functional relationships between hydraulic conductivity and ABA root signaling pathway, plasma membrane aquaporins (*Cs*PIPs) molecular regulation and some shoot water related parameters ($G_s$ and ABA in leaves). Chapter I.

2. To asses the relative role of SHA mediated PM-$H^+$-ATPase activity and hydraulic conductivity on shoot and/or root growth promotion of cucumber plants. Chapter II

3. To study the chemical nature and the molecular conformation of SHA fractions obtained form humeomic sequential fractionation. To provide SHA bulk structure information by a theoretical model based on molecular mechanics. To asses the behavior of the obtained fractions on plant development and their role on activating hydraulic conductivity and plant growth. Chapter III

4. To investigate SHA primary action in *Arabidopsis thaliana* seedlings under square plates growing conditions studying specifically the regulation of root genes associated with root growth and with any of the SHA promoted root event. Chapter IV.

# CHAPTER I:

**ABA-regulation of root hydraulic conductivity and aquaporin gene-expression is crucial to the plant shoot growth enhancement caused by rhizosphere humic acids**

***Pubished in Plant Physiology, 2015. dx.doi.org/10.1104/pp.15.00596***

**Abstract**

The physiological and metabolic mechanisms behind the humic acid-mediated plant growth enhancement are discussed in detail. Experiments using cucumber plants show that the shoot growth enhancement caused by a structurally well characterized humic acid with sedimentary origin is functionally associated with significant increases in ABA root concentration and root hydraulic conductivity. Complementary experiments involving a blocking agent of cell-wall pores and water root transport (polyethylenglycol, PEG) show that increases in root hydraulic conductivity is essential in the shoot growth promoting action of the model humic acid. Further experiments involving an inhibitor of ABA biosynthesis in root (fluridone, Fld) show that the humic acid-mediated enhancement of both root hydraulic conductivity and shoot growth depended on ABA-signaling pathways. These experiments also show that a significant increase in the gene expression of the main root plasma membrane aquaporins is associated with the increase of root hydraulic conductivity caused by the model humic acid. Finally, experimental data suggests that all these actions of model humic acid on root functionality, which are linked to its beneficial action on plant shoot growth, are likely related to the conformational structure of humic acid in solution and its interaction with the cell wall at the root surface.

## Introduction

### Two theories that conducted the present study

Many studies have reported that HAs obtained from either organic materials (soils, soil sediments, composted wastes …) or water reservoirs (rivers, lakes, …), extracted with alkaline water solutions or isolated by resin fixation, reverse osmosis or ultrafiltration (Alberts and Takács, 2004) affected the development of diverse plant species (for instance, cucumber, tomato, maize, wheat, Arabidopsis, rapeseed) through common signaling pathways, which involved key phytoregulators such as indole-acetic acid-nitric oxide (IAA-NO) (Zandonadi et al., 2010; Canellas et al., 2011; Trevisan et al., 2011; Mora et al., 2012, 2014a), ethylene and abscisic acid (ABA) in roots (Mora et al., 2012, 2014a), as well as cytokinins (CKs) in shoots (Mora et al., 2010, 2014b). Recently, Mora et al. (2014a) showed that the HA ability to enhance both shoot growth and ABA-root concentration in cucumber was regulated by NO (dependent or independent), IAA and ethylene root signaling pathways. However, despite all these information, the nature of a possible primary, common, physiological action on plant roots of HAs with diverse origin and structure remains elusive.

Recently, Asli and Neumann (2010) described a new mechanism by which high concentrations of HAs extracted from diverse organic sources decreased shoot plant growth. This mechanism involved the reduction of root hydraulic conductivity ($Lp_r$) resulting from the fouling of root cell wall pores due to the accumulation and aggregation of HA molecules at root surface. Even though the concentration of HAs used by Asli and Neumann (2010) (1 g $L^{-1}$) is much higher than that related to HA-plant growth promotion ability (50-250 mg $L^{-1}$) (Rose et al. 2014) their results do raise the hypothesis that the primary, still unknown, event emerging from the interaction of (humic substances) HS with root surface cells might involve an unspecific, physical action on root permeability and water uptake. This event might trigger a chain of secondary events in the root that in turn would affect specific hormone signaling pathways, which may regulate shoot- and root- growth. This HAs action on plant development would be positive (increasing) or negative (decreasing) depending on HAs concentration in the rhizosphere.

**Special mention to plant water relations**

Growth rate is typically linked to water and nutrient availability. Water uptake by the roots and further distribution within the plant contributes to the global water balance in plants. Even though this process is essential for plant survival, the precise mechanism of water transport in plants is still debated. Generally speaking, there are two terms that can be distinguished when we are referring to water uptake and transport. Long-distance transport and short distance transport (figure I, 1). Long-distance transport refers to xylem ascent through xylem vessels (capillaries made from dead cells). This movement is guided by hydrostatic pressures created by transpiration (water demand from leaves). Short-distance transport instead, refers to the regulation of cell water homeostasis and it is required to maintain long distance water transport because it contributes totally to it. In this mentioned short transport, water has to flow across different living tissues. In fact, Steudle and Frensch (1996) and Steudle and Peterson (1998) proposed a composite transport model (CMT) in which the different contributions for root water uptake are considered. Three different pathways of water transport have been described: the apoplastic path around the protoplasts, the symplastic path through the plasmodesmata and the transcellular path across the cell membranes which is tightly controlled by the amount and activity of water channels or aquaporins (figure I, 2). There are no experimental approaches to separate experimentally the symplastic and transcelullar components. Therefore, they are summarized as “cell-to-cell” paths. Each of the pathways contributes differently to the general water transport depending on the physical nature of the driving force. In the presence of hydrostatic pressure gradients (under transpiration), flow moves around the apoplastic path, in the presence of osmotic gradients (in the absence of transpiration during root exudation for example) however, water flows by the “cell-to-cell” path (Steudle and Frensch, 1996; Steudle and Peterson, 1998)

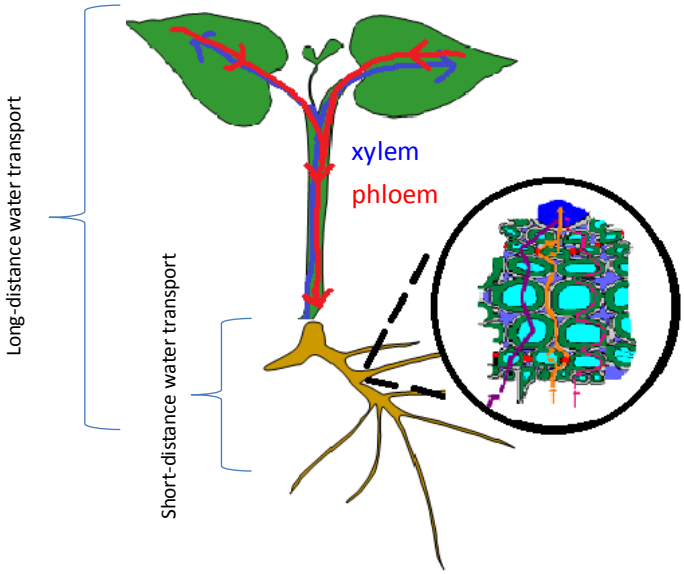


**Figure I, 1:** Short and long-distance water transport in plants.

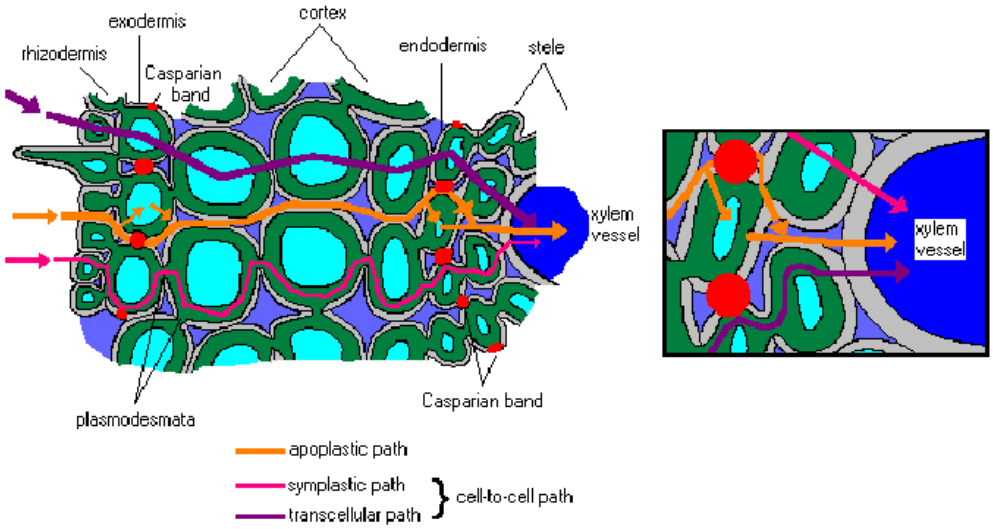


**Figure I, 2:** The apoplastic, symplastic and transcelullar pathways of water in roots. (Steudle, 2000).

When it refers to the quantification of water uptake in plants, the major parameter reflecting root water uptake ability is root hydraulic conductivity ($Lp_r$) (Martínez-Ballesta et al., 2011). In relation to the contribution of each of the described pathways to the general $Lp_r$, hydrostatic gradients (apoplastic path) may result in higher root hydraulic conductivity than osmotic gradients (Steudle, 2000). However, this behavior is not always observed.

Progress in the anatomical structure of the root at molecular level, particularly through aquaporins activity, has broadly developed the study of root water uptake and general plant hydraulics (Steudle, 2000). Aquaporins belong to a large family of water channel proteins located in plasma and intracellular membranes called Mayor Intrinsic Proteins (MIP). They can be divided into different subfamilies depending on their features of sequence homology and subcellular location: Plasma Membrane Intrinsic Proteins (PIPs), the Tonoplast Intrinsic Proteins (TIPs), the Nodulin-26-like Intrinsic Proteins (NIPs), the Small Basic Intrinsic Proteins (SIPs) and the uncategorized X Intrinsic Proteins (XIPs) (Javot et al., 2003; Maurel et al., 2008; Li et al., 2014).

Regulation of aquaporins was observed at transcriptional, post-translational level and process involving targeting of aquaporin.

Concerning plant aquaporing gating mechanisms (the opening and closing of the water channel pore) the influence of protons ($H^+$), divalent cations (being $Ca^{+2}$ the most efficient) and $H_2O_2$ (one of the most important ROS) on changing the locked or unlocked state of the water channel are the most studied effectors.

Post-translational modifications involve phosphorilation of one of the residues of aquaporin structure or methylation events as the most studied factors (Horie et al., 2011).

In relation to hormone and environmental related regulation, highly expressed aquaporin members in Arabidopsis were reported to be affected by salt stress and ABA (sometimes dependent or independent pathways), indicating that the expression of aquaporins is affected by varieties, severity of stressors, duration of treatment and species or developmental stage of plants (Zhu, 2005).

However, the regulation in activating or down-regulating aquaporin activity is very extensive and changeable and it needs further studies (Li et al., 2014)

In the present study, the involvement of HA on plant water relations has been studied. Specifically, the osmotic contribution of $Lp_r$ to plant water uptake was measured, which means the contribution of "cell to cell" path and the activation of aquaporins in this process. Thus, four of the most relevant PIPs were measured: *Cs*PIP 1;2, *Cs*PIP 2;1, *Cs*PIP 2;4 and *Cs*PIP 2;5.

**Recent advances on ABA biosynthesis roles within the plant**

The most common role of ABA synthesis in chloroplasts responds to closing stomata and reducing transpiration and leaf expansion in water scarcity. However, it is changing the traditional view that this hormone is generally involved in growth inhibition (Sharp, 2002). In fact, an important role of endogenous ABA is to limit ethylene production (mainly in water stressed plants) and maintain root elongation (Sharp and LeNoble, 2002). In this line, ABA itself can increase the flow of water into and through the root, activating $Lp_r$ (Hose et al., 2000). This is believed to result from ABA-induced opening of these inwardly directed water channels (Tyerman et al., 1999). Regarding ABA involvement in shoot, ABA induces increases of various second messengers such as cytosolic $Ca^{2+}$, reactive oxygen species (ROS) and nitric oxide (NO) in guard cells. These early signal components finally evoke ion efflux through plasma membrane ion channels, resulting in reduction of guard cell turgor pressure (Munemasa et al., 2007)

Additionally, it is important to note that all tissues of maize roots, regardless of their age and position, could synthesize ABA when dried and it might be that this above described ABA derived events could also happen in other tissues taking into account that sometimes the regulation of some physiologic responses involves crosstalk between shoot and root (Zhang and Tardieu, 1996).

**Main prospects of study**

Based on recent works published by Mora et al. (2014a) in which SHA action involves ABA synthesis in root and the relationship between ABA and $Lp_r$ activation in roots, we

have tested the potential role of root hydraulic conductivity in the main mechanism by which HAs promote shoot growth in cucumber (*Cucumis sativus* L. cv Ashley). To this end, we used a well-characterized and modeled sedimentary HA (SHA) at a concentration (100 mg-SHA organic carbon (C) $L^{-1}$) that was associated with plant shoot growth promotion in previous studies (Mora, 2009; Mora et al., 2014a,b). We also investigated the functional relationships between these effects of SHA on root hydraulic conductivity and shoot growth as well as in some shoot water related parameters ($G_s$ and ABA), and those caused by SHA on (IAA/NO) and ABA root signaling pathways. Finally, taking into account that root plasma membrane aquaporins (PIPs) are involved in the ABA-regulation of root hydraulic conductivity in other plant systems we also studied the role of root plasma membrane aquaporin (PIPs) molecular regulation in SHA effects on plant shoot growth.

The results obtained here show that SHA enhances shoot growth in cucumber through ABA-dependent increases in both root hydraulic conductivity and root PIPs (*Cs*PIPs) gene up-regulation.

**Material and Methods**

**Extraction and purification of a leonardite humic acid (SHA)**

The sample of HA obtained from leonardite (Danube basin) was used in the experiments. A specific amount of HA (100 g) was extracted, and purified using the IHSS methodology (http://www.humicsubstances.org/soilhafa.html) as described in Aguirre et al., (2009).

**Plant material and culture conditions**

Seeds of cucumber (*Cucumis sativus* L. cv Ashley) were germinated in water with 1 mM of $CaSO_4$, in darkness, on perlite and moistened filter paper in a seed germination chamber. One week after germination, plants were transferred to 8 L recipients in hydroponic solution. The nutrient solution used was: 0.63 mM $K_2SO_4$; 0.5 mM $KH_2PO_4$; 0.5 mM $CaSO_4$; 0.30 mM $MgSO_4$; 0.25 mM $KNO_3$; 0.05 mM KCl and 0.87 mM $Mg(NO_3)_2$; 40 µM $H_3BO_3$; 4 µM $MnSO_4$; 2 µM $CuSO_4$; 4 µM $ZnSO_4$ and 1.4 µM $Na_2MoO_4$. The nutrient solution contained 40 µM of iron as Fe-EDDHA chelate (80 % ortho-ortho

isomer). No precipitation of Fe inorganic species was observed throughout the experiment. The pH of the nutrient solutions was held at 6.0 and did not change significantly during the experiment. All experiments were performed in a growth chamber at 25/21 ºC, 70-75% relative humidity and with 15/9 h day/night photoperiod (irradiance: 250 µmol $m^{-2}$ $s^{-1}$), $CO_2$ content 400 ppm.

At these conditions, three different experiments were undertaken:

1. General effects of SHA on water balance. After ten days of growth in hydroponics, SHA treatment (100 mg $L^{-1}$ of organic carbon) was applied to plants. The following physiological parameters were measured: shoot relative growth rate (SRGR), stomatal conductance ($G_s$), ABA concentration in leaves, $Lp_r$ and water content in leaves by THzA.

2. Effect of SHA action on water root uptake using a water uptake inhibitor (PEG). After growing plants in the conditions described above during 10 days, the following treatments were applied: Control with nutrient solution but without any treatment, SHA treatment (100 mg $L^{-1}$ of organic carbon), Polyethylene glycol (PEG) treatment (100 mg $L^{-1}$ PEG-organic carbon or 10 % PEG in another experiment series) and a treatment combining PEG and SHA (PEG+SHA). Polyethylene glycol ($M_r$) 20,000 reagent was purchased from Sigma-Aldrich and the quantity needed for each treatment was dissolved into the nutrient solution. The choice of the molecular weight of PEG was done according to both the size of root cell-wall pores in cucumber and its similarity to SHA apparent size.

3. Involvement of ABA balance in roots on water root uptake. A well-known ABA biosynthesis inhibitor, Fluridone, was used to perform this experiment (Chae et al., 2004). After growing plants in the conditions described above during 10 days, the following treatments were applied: Control with nutrient solution but without any treatment, SHA treatment (100 mg $L^{-1}$ of organic carbon), Fluridone (Fld) treatment (10µM of Fld) and a treatment combining Fld (10µM) and SHA (100 mg $L^{-1}$ of organic carbon) (Fld+SHA). The concentration of fluridone was based on experiments reported by Sánchez-Romera et al., (2014) and dose-

response experiments were carried out before conducting final experiments. We analyzed shoot growth, $Lp_r$, ABA concentration in roots and aquaporin gene expression.

All treatments involved a minimum of five replications (five plants per treatment). Harvests were conducted at the same time of the day to exclude diurnal light variations, this meant 6 h after the start of the light period. Plants were harvested at 4, 24, 48 and 72 h after the application of treatments. One part of the plant material was weighed and dried (60 °C) for Shoot Relative Growth Rate (SRGR) determination, and another part was frozen in liquid nitrogen and stored at -80 °C for further analysis. Shoot relative determination was calculated as explained by Hoffmann and Poorter (2002).

SRGR= ( $\overline{\ln W2}$ – $\overline{\ln W1}$) / ($T_2$-$T_1$)

Where:

ln = natural logarithm

$T_1$ and $T_2$ time of harvests (in days)

$W_1$ and $W_2$ = dry weight of shoot at time one and time 2.

**Measurement of root hydraulic conductivity ($Lp_r$) in the absence of hydrostatic pressure gradients (free exudation method) ($Lp_r$)**

Root exudates collection: the collection of xylem sap is based on “root pressure” and water follows a “cell-to cell” pathway (Gibbs et al., 1998a; Gibbs et al., 1998b). The interest of using this methodology is that the regulation of “cell-to cell” water transport involves plasma membrane aquaporin activity (Maurel et al., 2008), a parameter that was also evaluated in experiments. Further, previous studies showed the good concordance between $Lp_r$ measured by free exudation method and that measured by pressure chamber method (Sanchez-Romera et al., 2014)

Plants were excised in their growing hydroponic medium. Stems were first cut from just below the first leave. Then, the top part of the stem was introduced in a silicone tube and sealed with a self-sealing film to avoid any loss of sap.

Root exudates were finally collected with a glass Pasteur pipette. Collections were done continuously during the first 90 minutes of exudation at 4, 24, 48 72 hours of treatment and kept in a previously weighted 1.5 mL tube.

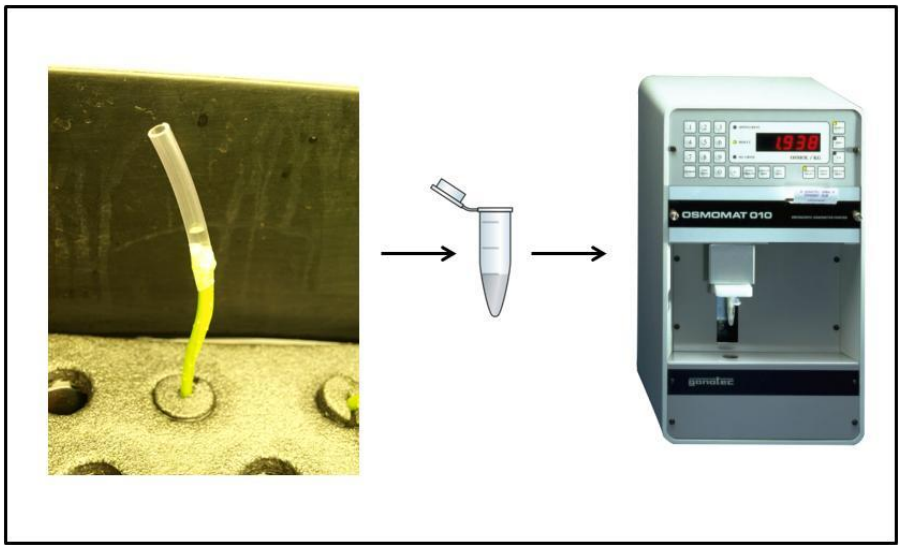


**Figure I, 3:** Plant root exudation process followed by sap collection in tubes and final osmotic pressure measurement.

Measurement of the osmotic pressure of exuded sap: the osmolality of root exudates was measured using a freezing point depression osmometer (Osmomat 010 Gonotec, Germany). Osmolality (mOsmol) was converted to osmotic pressure (MPa) according to a described procedure (Bigot and Boucaud, 1998).

$MPa = mOsmol \times 0.831 \times 10^{-5} \times T(°K)$

In the absence of hydrostatic pressure gradients, water uptake by the root is governed by the differences in osmotic pressure between the medium and the sap (Miyamoto et al., 2001). The equation to calculate hydraulic conductivity ($Lp_r$) is described as:

$Jv = Lp_r \times \sigma_{sr} \times \Delta\pi$

The coefficient $\sigma_{sr}$ denotes the reflection coefficient of solutes in the roots, which was reported to be 0.853 by previous studies (Miller, 1985). Thus, we could measure root hydraulic conductivity ($Lp_r$) by measuring water flow and pressure differences ($\Delta\pi$).

Osmotic potential of solutions containing either SHA or PEG was also evaluated.

**Water distribution in leaves evaluated using Active THz imaging-system Analysis (THzA)**

THz imaging-system Analysis THzA has been utilized in both medical applications (Pickwell-MacPherson and Wallace, 2009; Smith and Arnold, 2011) and more recently, in water content determination in leaves (Castro-Camus et al., 2013; Born et al., 2014; Gente and Koch 2015). Water has a typical resonance that can be measured when it is applied a frequency between 0.14 and 0.22 THz. Thus, water content may be directly measured by this range frequency analysis (Santesteban et al., 2015). The imaging set-up system used is based on a E8361C PNA Agilent Network Analyzer. In order to work in THz range, two OML Vector Network Analyzer (VNA) Extenders, working in the frequency range from 0.14 THz to 0.22 THz have been connected to the analyzer. A pair of plane-convex lenses has been included to increase resolution. A 3D scanning system has been used for mapping the leaves. The 3D scanning system is moved in the xz plane for a fixed y-position, in order to map the whole leaf. Each measurement point is corresponded with a pixel in the final THz image, and presents information about the attenuation through the leaf due to water content. The movement of the 3D scan and the data acquisition is governed by an in house LabView interface program. The system dynamic range is higher than 60 dB for all the bandwidth.

**Analysis of the concentration of abscisic acid (ABA) in plants tissues**

The concentration of ABA was analyzed using HPLC/MS/MS as described below.

The extraction and purification of ABA were carried out using the previously described methods (Dobrev and Kaminek, 2002; Aguirre et al., 2009).

Liquid Chromatography-Mass Spectrometry quantification of ABA: the hormone was quantified by HPLC linked to a 3200 Q TRAP LC/MS/MS system (Applied

Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using an reverse-phase column (Synergi 4µm Hydro-RP 80A, 150x2 mm, Phenomenex, Torrance, CA). A linear gradient of methanol and 0.5% acetic acid in water was used: 35% methanol; time 1 min, 35%-95%methanol; time 9 min, 95% methanol; time 4 min and 95%-35% methanol; time 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 mL/min, the injection volume was 50 µL and the column and sample temperatures were 30 and 20 ºC, respectively.

Detection and quantification were performed by MRM in the negative-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standards. The source parameters are: curtain gas: 25.0 psi, GS1: 50.0 psi, GS2: 60.0 psi, ion spray voltage: -4000 V, CAD gas: medium, and temperature: 600 ºC.

**Reverse transcription polymerase chain reaction (RT PCR) analysis of RNA transcript**

The roots of the plants were collected and disrupted with liquid nitrogen prior to RNA extraction. Total RNA was extracted from between 50 and 90 mg of crushed root using a mix of 350 µL of guanidinium-thocyanate lysis buffer and 3.5 µL of β-mercaptoethanol of NucleoSpin RNA Plant Kit (Macherey-Nagel, Düren, Germany). Following treatment of RNA with DNase was performed according to the manufacturer`s recommendations. After washing extracted RNA with dry silica membranes provided by the kit, RNA purity and concentration was quantified by fluorescence-based Experion RNA STdSens Analysis kit. First-strand cDNA synthesis was carried out in 20-µL reactions containing 1 µg of RNA with RNase H+ MMLV reverse transcriptase iScript and a mix of oligo(dT) and random hexamer primers from iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The reverse transcription was made up for 5 min at 25 °C, 30 min at 42 °C, and ended by 5 min at 85 °C. Reverse transcription PCR was performed using iQ SYBR Green supermix containing hot-start iTaq DNA polymerase in an iCycler iQ (Bio-Rad Laboratories). Primer pairs used to amplify cucumber plasma membrane aquaporin were taken from previously published work (Qian et *al*. 2014) and synthesized by Sigma-Genosys (Cambridge, United Kingdom). Standarization was carried out based on the expression

of the *Cucumis sativus* cyclophilin (CYP) gene in each sample, using corresponding specific primers (Acc. No. AY942800).

The RT PCR program consist of an iTaq DNA polymerase activation at 95 °C for 3 min, followed by 40 amplification cycles (denaturing step for 10 s at 95 °C; an annealing step for 10 s at 62 °C, and an elongation step for 10 s at 72 °C during which the fluorescence data were collected). To confirm PCR products, a melting curve was performed by heating the samples from 55.8 to 65 °C in 0.2 °C increments with a dwell time at each temperature of 10 s during which the fluorescence data were collected. The melting temperature of the products was determined with the iCycler iQ Optical System 3.1 Software (Bio-Rad Laboratories). Data analysis of the relative abundance of the transcripts was done using CFX Manager Software Data Analysis (Bio-Rad Laboratories). Expression analyses were carried out in five independent root RNA samples and repeated three times for each RNA sample. The Ct values for cyclophilin reference gene ranged among 18.65 for the lowest value and 20.10 for the highest one for all the treatments at all harvest times.

**Statistical analysis**

Significant differences ($p < 0.05$) among treatments were calculated by using one-way analysis of variance (ANOVA) and the LSD Fisher *post hoc* test. All statistical test were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa USA). All the error bars represent the standard error of each of the measurements. The mean values of the measured parameters were calculated with (n=5) independent samples. Only $Lp_r$ was calculated with n=7 independent samples.

## Results

### The growth promoting action of SHA in cucumber is associated with a significant increase in root hydraulic conductivity ($Lp_r$) and leaf stomatal conductance ($G_s$)

In order to determine whether the SHA-mediated enhancement in shoot growth is associated with changes in $Lp_r$, we studied the effect of the application of 100 mg C-SHA $L^{-1}$ on both parameters in cucumber plants.

In line with previous studies, cucumber plants treated with 100 mg C-SHA $L^{-1}$ experienced a significant increase in the shoot relative growth rate (SRGR) with respect to non-treated control plants (table I, 1): 186 mg $g^{-1}$ $d^{-1}$ versus 133 mg $g^{-1}$ $d^{-1}$ respectively. This increase of SRGR caused by SHA was associated with a significant increase in $Lp_r$ (Figure I, 4).

In line with above-mentioned results, SHA caused a significant increase in $G_s$ after 4 and 24 hours from the onset of the treatment (figure I, 5).

This effect was consistent with the variation of ABA concentration in leaves, which showed no significant differences between treatments for all harvest times (figure I, 6).

**Table I, 1.** Shoot Relative Growth Rate for SHA treated plants.

| | **SRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|
| **C** | 133 b |
| **SHA** | 186 a |
| $p < 0.05$ (Fisher) | |

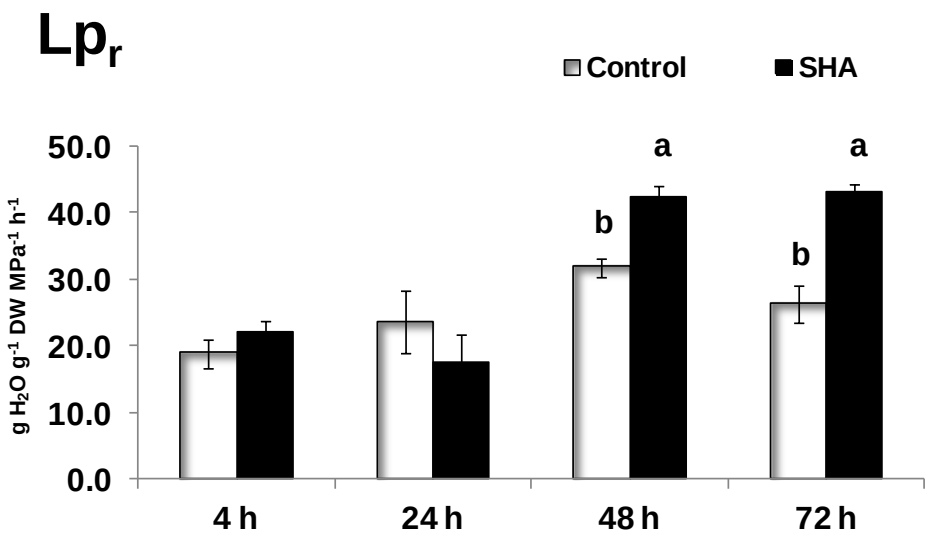


**Figure I, 4:** $Lp_r$ ± SE (n=7) values for control and sedimentary humic acid (SHA) treated plants measured at each harvest time between 4 and 72 hours.

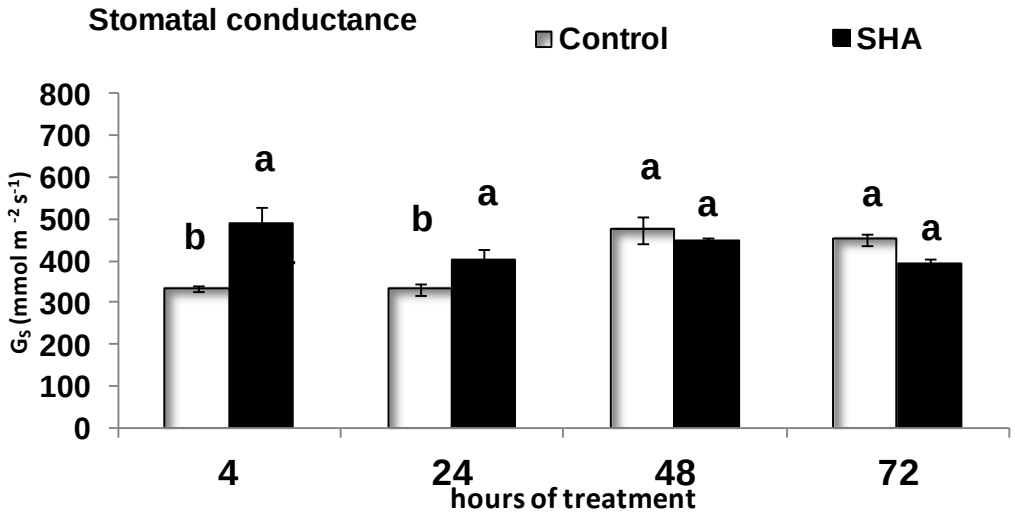


**Figure I, 5**: Stomatal conductance of control and SHA treated plants after 4, 24, 48 and 72 hours of treatment. Different letters mean significant differences between treatments. ($p<0.05$).

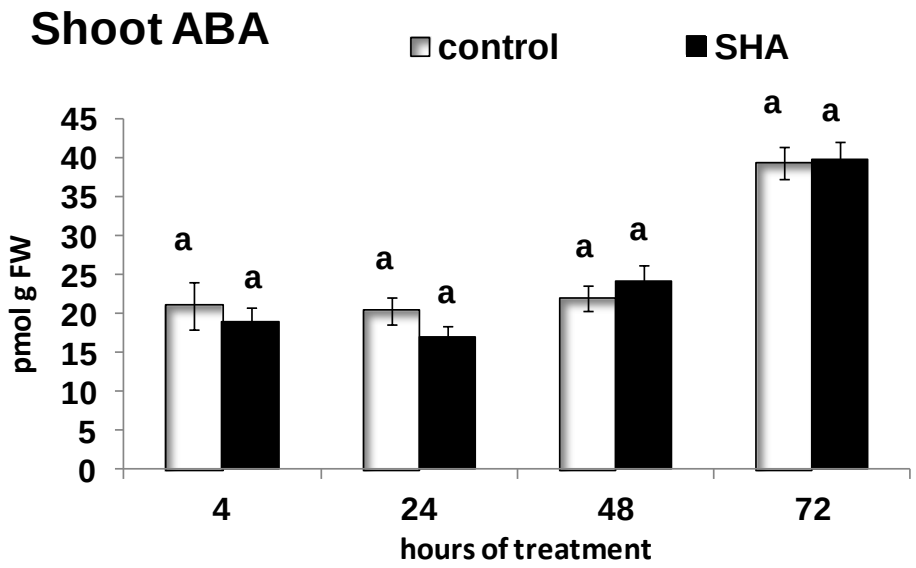


**Figure I, 6:** Shoot ABA concentration for control and SHA treated plants. Different letters mean significant differences between treatments. ($p<0.05$).

**SHA was able to reverse the negative effect of 100 mg C $L^{-1}$ of polyethyleneglicol (PEG) on both $Lp_r$ and plant shoot growth, with this effect being associated with an increase in both ABA root concentration and $Lp_r$**

Asli and Neumann (2010) reported that high concentrations of diverse sedimentary humic acids and PEG (1 g $L^{-1}$) caused a decrease in $Lp_r$ and SRGR derived from fouling in root cell wall pores.

In order to determine whether both the shoot growth promoting effect and the increase in $Lp_r$ of 100 mg-C $L^{-1}$ SHA is also shared by similar concentrations of PEG, we studied the effect of 100 mg C-PEG $L^{-1}$ on both SRGR and $Lp_r$ values in cucumber plants. The results showed that 100 mg C-PEG $L^{-1}$ caused a significant reduction in $Lp_r$ at 4, 24 and 72 h upon treatment (figure I, 7). This fact was also associated with a significant decrease in SRGR in comparison with both SHA-treated and non-treated plants (table I, 2). Yet, no differences between the osmotic potential of solutions containing either SHA or PEG were found.

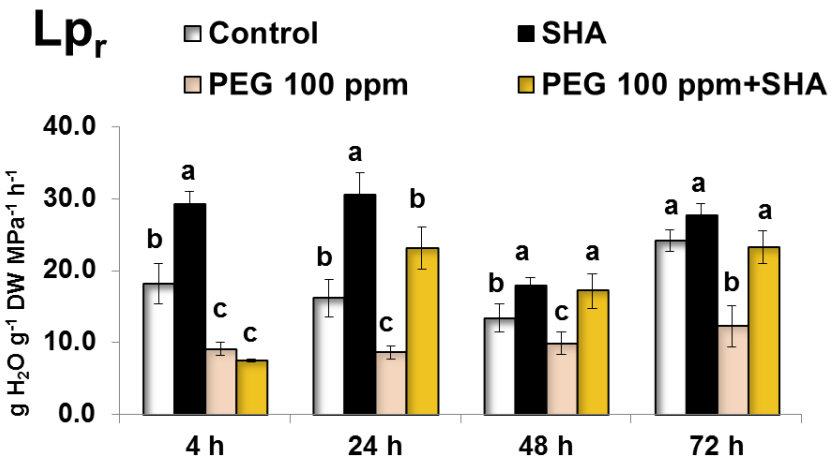


**Figure I, 7:** $Lp_r$ ± SE (n=7) values for control, sedimentary humic acid (SHA), polyethylene glycol (PEG) and both PEG+SHA treated plants measured at each harvest time between 4 and 72 hours.

**Table I, 2.** Shoot Relative Growth Rate for PEG and PEG+SHA treated plants between 4 and 72 hours.

| | **SRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|
| **C** | 113 b |
| **SHA** | 144 a |
| **PEG** | 98 c |
| **PEG+SHA** | 140 a |
| p < 0.05 (Fisher) | |

The addition of 100 mg C-SHA $L^{-1}$ to PEG-treated plants reversed the negative effect of PEG on both $Lp_r$ and SRGR at 24, 48 and 72 h upon treatments (figure I, 7, table I, 2). However, it is important to note that similar experiments but using 10 % PEG for plant treatments showed that the addition of 100 mg C $L^{-1}$ SHA was not able to reverse the

negative action of 10 % PEG on SRGR (table I, 3). This fact that was associated with a significant increase in leaf-ABA concentration for both 10 % PEG and 10% PEG plus 100 mg C $L^{-1}$ SHA (table I, 4).

**Table I, 3.** SRGR of plants treated with SHA after the application of higher concentration of PEG (10%).

| | **SRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|
| **C** | 235 b |
| **SHA** | 350 a |
| **PEG 10 %** | 205 c |
| **PEG 10 %+SHA** | 214 bc |

p< 0.05 (Fisher)

**Table I, 4.** Leave Abscisic acid (ABA) concentration (pmol $g^{-1}$ FW (fresh weight)) for control, SHA, PEG 10% and both PEG 10%+SHA treated plants measured at each harvest time between 4 and 72 hours.

| | **Hours** | | | |
|---|---|---|---|---|
| | **4** | **24** | **48** | **72** |
| **C** | 40.0 bc | 36.7 b | 38.1 c | 47.2 c |
| **SHA** | 32.2 c | 47.3 b | 34.1 c | 47.6 c |
| **PEG 10 %** | 63.1 ab | 129 a | 81.3 b | 180 b |
| **PEG 10 %+SHA** | 83.2 a | 162 a | 115 a | 244 a |

p> 0.05 (Fisher)

The above mentioned effects of SHA in PEG-treated plants were consistent with SHA-induced changes in root ABA concentration. Whereas 100 mg C $L^{-1}$ PEG treated plants did not show any sustained change in root ABA concentration with respect to control plants (only a slight decrease at 24 h), a sustained increase in this parameter was observed for SHA-treated plants and for plants treated with 100 mg C $L^{-1}$ PEG+SHA (figure I, 8). Nevertheless, while the root ABA-increase in SHA-treated plants is expressed even from 4 hours upon SHA addition, in PEG+SHA treated plants this effect needed more time to be expressed, being significant after 24, 48 and 72 h of treatment application (figure I, 8). In any case, a good concordance between the changes in root ABA concentration and those related to both water root uptake ($Lp_r$) and SRGR was observed for all treatments (figure I, 7, table I, 2).

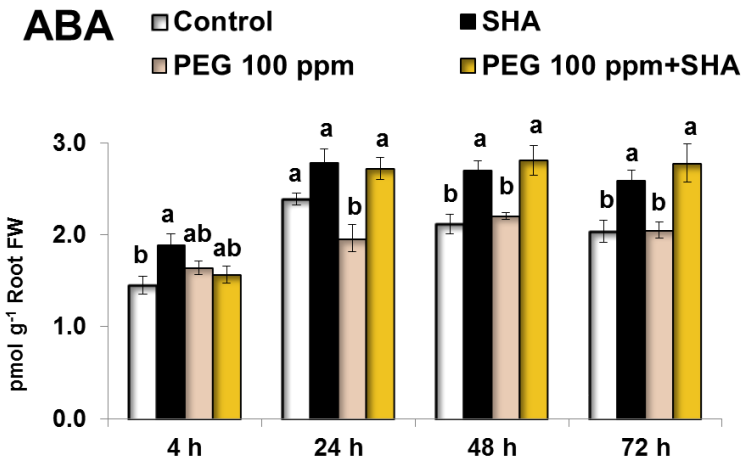


**Figure I, 8:** Root abscisic acid (ABA) concentration (pmol $g^{-1}$ FW (fresh weight)) ±SE (n=5) for control, sedimentary humic acid (SHA), polyethylene glycol (PEG) and both PEG+SHA treated plants measured at each harvest time between 4 and 72 hours. Different letters means statistical differences at each harvest time.

**The promoting action of SHA on both $Lp_r$ and shoot growth is expressed through ABA-dependent pathways**

The ability of ABA in roots to enhance $Lp_r$ and hence to improve plant water status has been demonstrated in some studies (Hose et al., 2000; Thompson et al., 2007; Tardieu et al., 2010). In order to check whether the enhancement in both $Lp_r$ and shoot growth caused by SHA is functionally dependent of the observed SHA-mediated increase in ABA root concentration, the effect of an inhibitor of ABA biosynthesis (Fluridone, Fld) on these actions of SHA was studied in cucumber plants.

Fld application caused a significant decrease in ABA root concentration in plants either treated or non-treated with SHA (figure I, 9). In non-treated plants, Fld also caused a significant and sustained reduction in $Lp_r$ (figure I, 10). Likewise, the SHA-mediated increase in $Lp_r$ was removed when SHA was applied along with Fld (figure I, 10).

In addition, the shoot growth promoting action of SHA, which was reflected in a significant increase in SRGR (table I, 5), disappeared for those plants treated with both Fld and SHA along with Fld.

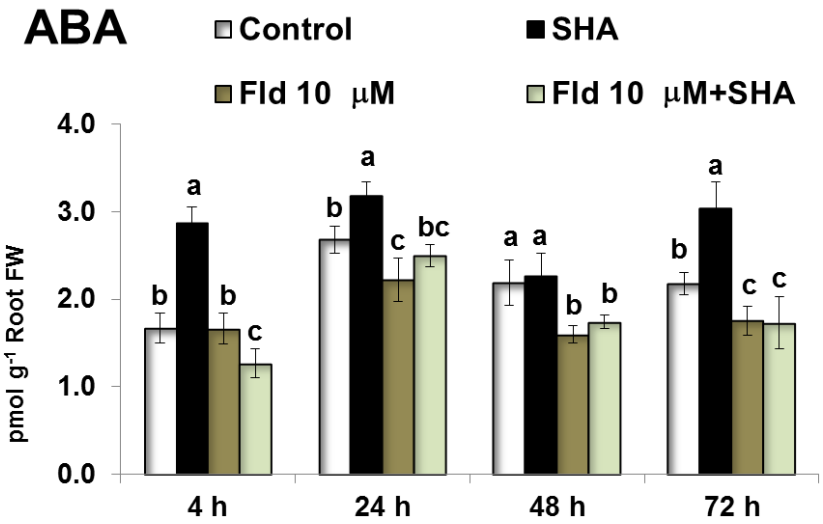


**Figure I, 9:** Root abscisic acid (ABA) concentration (pmol $g^{-1}$ FW (fresh weight)) ±SE (n=5) for control, sedimentary humic acid (SHA), fluridone (Fld) and fluridone + sedimentary humic acid (Fld+SHA) treated plants measured at each harvest time

between 4 and 72 hours. Different letters means statistical differences at each harvest time.

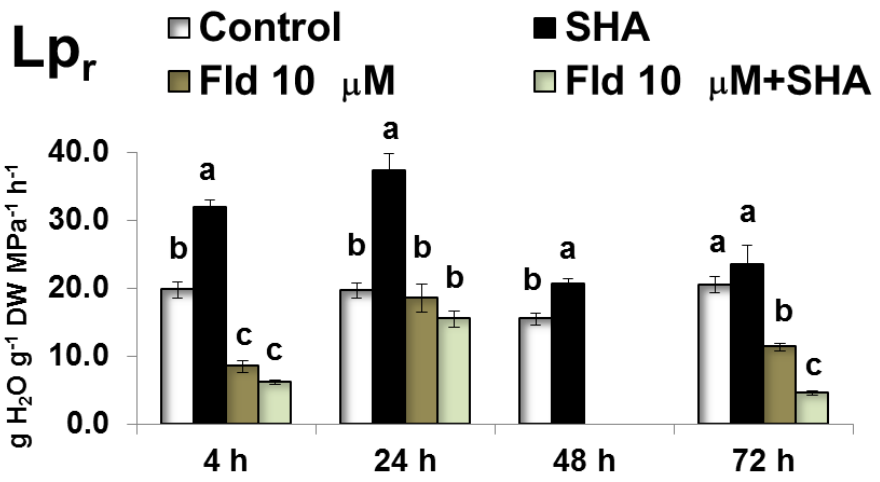


**Figure I, 10:** $Lp_r$ ±SE (n=7) values for control, sedimentary humic acid (SHA), fluridone (Fld) and fluridone + sedimentary humic acid (Fld+SHA) treated plants measured at each harvest time between 4 and 72 hours. At 48 hours of treatment, exudation did not occur for Fld and SHA+Fld treated plants and measurements of $Lp_r$ could not be completed. Different letters means statistical differences at each harvest time.

**Table I. 5:** Shoot Relative Growth Rate for Fld and Fld+SHA treated plants between 4 and 72 hours.

| | SRGR (mg $g^{-1}$ $d^{-1}$) |
|---|---|
| C | 113 b |
| SHA | 170 a |
| Fld | 88.5 b |
| Fld+SHA | 78 b |

$p < 0.05$ (Fisher)

**SHA application increased the expression of root plasma membrane aquaporin (CsPIPs) gene expression through ABA- dependent and independent pathways**

A number of studies have reported that the regulation of $Lp_r$ by ABA is functionally related to the increase in the expression of the genes encoding several root plasma membrane intrinsic proteins (PIPs) named aquaporins (Quintero et al., 1999; Beaudette et al., 2007; Vandeleur et al., 2014). In order to investigate if the ABA-mediated increase in Lpr caused by SHA is also linked to a concomitant increase in the expression of genes encoding *Cs*PIPs, the effect of SHA on *Cs*PIPs gene expression was measured with and without the presence of Fld. Those *Cs*PIPs that presented larger gene expression in cucumber were selected for the study: *Cs*PIP1;2 21.7%, *Cs*PIP2;1 6.1%, *Cs*PIP 2;4 59.4% and *Cs*PIP 2;5 6.5% (Qian et al., 2014). The results showed that SHA treatment caused a significant up-regulation of all *Cs*PIPs studied genes with this action depending on *Cs*PIPs. Thus, SHA caused a significant increase in the gene expression of *Cs*PIP 1;2, *Cs*PIP 2;4 and *Cs*PIP 2;5 at 24, 48 and 72 h (figure I, 11), while the gene expression of *Cs*PIP 2;1 increased only after 4 h upon SHA application (figure I, 11). It is noticeable the low level of gene expression of *Cs*PIP 2;1 in either control or SHA-treated plants.

Experiments including Fld treatment in plants either treated or non-treated with SHAs showed that Fdl per se significantly increased *Cs*PIPs expression, with the time-course of this effect being dependent on the *Cs*PIP type (figure I, 11).

On the other hand, plants treated with both SHA and Fld presented *Cs*PIPs gene expression patterns very similar to those of plants treated with only Fld, except for *Cs*PIP1,2 after 48 h and *Cs*PIP2,5 after 72h, which presented an expression that was significantly higher than the other treatments: control, SHA-treated and Fld-treated plants (figure I, 11).

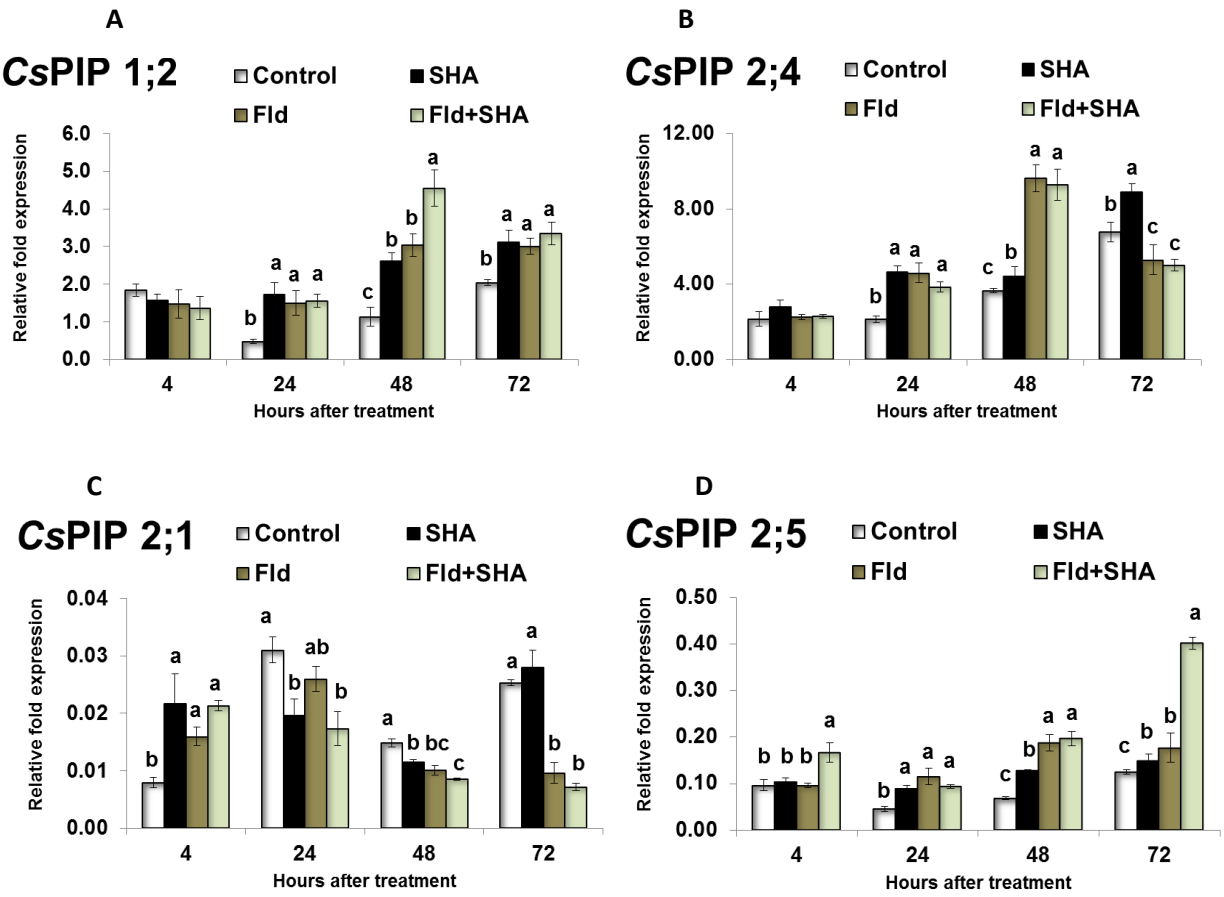


**Figure I, 11**: Relative aquaporin gene expression ±SE for the follollowing treatements: control, sedimentary humic acid (SHA), fluridone (Fld) and fluridone + sedimentary humic acid (Fld+SHA). A: Relative expression ±SE of CsPIP1;2 gene in cucumber roots. B: Relative expression ±SE of CsPIP 2:4 gene in cucumber roots. C: Relative expression ±SE of CsPIP 2:1 gene in cucumber roots. D: Relative expression ±SE of CsPIP 2:5 gene in cucumber roots.

**Treatments did not affect water distribution in leaves**

Water distribution in plants can change under different external stimuli, particularly when these stimuli can modify plant water homeostasis (Castro-Camus et al., 2013). As regards an eventual effect of SHA on water local distribution in leaves (figure I, 12), the results showed that, as in the case of control non-treated plants, water tended to accumulate in nervations, forming concentric lines around leaf-basis. So, there were no

differences between control and SHA treatment concerning water distribution in leaves.

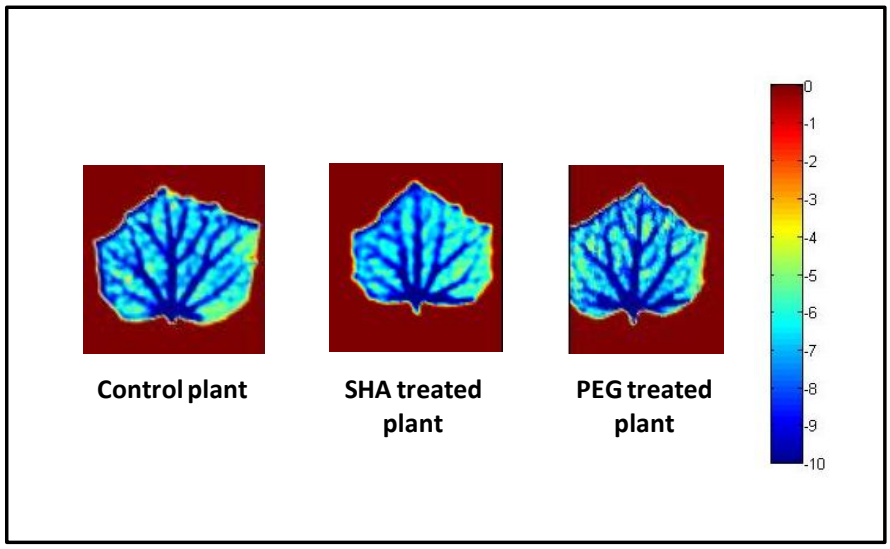


**Figure I, 12**: Water content distribution in leaves measured by Active THz imaging-system Analysis (ThzA) at 24 hours after the onset of treatment. From red to blue color means from less to more water leaf content.

**Discussion**

Current knowledge about the mechanism involved in the plant shoot promoting action of DOM (and HS present in DOM) is rather fragmentary and poorly integrated into a consistent explicative model (Nardi et al., 2009; Trevisan et al., 2011; Berbara and García, 2014; Canellas and Olivares, 2014; Mora et al., 2014b). Indeed, the fact that HAs with diverse origin and structural features affect plant growth and development through common signaling pathways remains unexplained. Recently, Asli and Neumann (2010) explained the negative effect on plant shoot growth of high concentrations of different types of HAs as a result of a reduction in root hydraulic conductivity caused by fouling of root-cell wall pores. Stemmed from these findings, the present study investigates whether the growth promoting action of HAs might also be linked to the interaction of HAs with cell walls at root surface. This type of physical action, rather unspecific, might explain why HAs with diverse structural features share common mechanisms to enhance plant growth. Thus, the character of the effect of HAs on plant growth, enhancement or inhibition, would be modulated by the HAs concentration in the rhizosphere. To this goal, we studied the effect of a well-characterized and modeled sedimentary-HA (SHA) on root water transport-related parameters but using a concentration in the nutrient solution able to promote shoot growth (100 mg as organic carbon $L^{-1}$) in cucumber plants.

In line with the study carried out by Asli and Neumann (2010), previous studies showed that high concentrations of SHA (0.80-0.85 g $L^{-1}$) in the rhizosphere caused slight (Azcona et al., 2011) or clear (Pascual et al. Personal Communication; Goicoechea et al. Personal Communication) decreases in the shoot growth of pepper and maize. In maize these results were linked to changes in water plant homeostasis and water stress induction (Goicoechea et al. Personal Communication). Similar results were obtained in cucumber and Arabidopsis (Mora, 2009) when high concentrations of SHA (0.80- 0.85 mg $L^{-1}$) were used. However lower concentrations of SHA (50-250 mg C-SHA $L^{-1}$) improved shoot and root growth in several plant species (Mora, 2009). In the present study a lower concentration of SHA (100 mg C $L^{-1}$) caused a significant increase in plant shoot growth that was reflected in SRGR values (table I, 1). This result was in line with previous results obtained using the same concentration of

sedimentary HAs in cucumber and rapeseed (Mora et al., 2010; Jannin et al., 2012). Furthermore, the present study also showed that the beneficial effect of SHA on shoot growth was accompanied by significant increases in both $Lp_r$ and $G_s$ (figure I, 4 and figure I, 5). These results, taken together with those from Asli and Neumann (2010), strongly suggest that both positive and negative effects on shoot growth resulting from SHA application involve an alteration of $Lp_r$ and hence root water transport. The SHA-action on shoot growth would be beneficial (increase) or detrimental (decrease) depending of SHA concentration (higher or lower than a specific threshold) in the rhizosphere.

Regarding the potential mechanism responsible for these differential effects of SHA on $Lp_r$ depending on SHA concentration level, Asli and Neumann (2010) related the shoot growth inhibition caused by high HAs concentration (1 g $L^{-1}$) to a primary action of HAs on root surface to some type of fouling of wall cell pores at root surface, which in turn caused decreases in $Lp_r$ and plant shoot growth. Considering that fouling is directly related to molecular size and molecular conformation in solution (compact, linear, flexible…), it is possible that the different effect of SHA is linked to some kind of effect of SHA concentration in solution (1 g $L^{-1}$ versus 0.1 g $L^{-1}$) on its molecular conformation and/or size. However, only SHA is affected in this way by concentration changes since PEG used at the same concentration as that of SHA (100 mg C $L^{-1}$), caused a sustained reduction of both $Lp_r$ and shoot growth (figure I, 7, table I, 2), as in the case of higher PEG concentrations. Numerous studies indicated that while intensity of fouling is affected by molecular concentration, its presence is only affected by molecular size (Hong and Elimelech 1997; Seidel and Elimelech 2002; Neumann et al., 2010). In consequence, the different results obtained when using low concentrations of PEG (detrimental) or SHA (beneficial), on shoot growth might be related to some specific, unshared, molecular feature of PEG and SHA. In this sense, while PEG is a linear, uncharged polymer, without tendency to aggregate in solution and settled into a random coil conformation (Oelmeier et al., 2012), SHA is a complex biomolecular system involving molecular aggregates and supramolecular conformations (Wershaw 1999; Piccolo 2002; Baigorri et al., 2007). Thus, whereas a reduction in concentration in solution does not affect PEG size, it might change SHA molecular aggregation and

hence SHA molecular size. This different molecular behavior for PEG and SHA under conditions of high or low concentration in solution might explain why, when concentration decreased fouling caused by PEG on wall cell pores remained while that associated with SHA disappeared. However, specific studies devoted to analyze SHA molecular size changes resulting from SHA-root surface (proton extrusion, root exudates and so on…) interaction must be carried out in order to know this phenomenon more in depth.

Interestingly, the application of 100 mg C-SHA $L^{-1}$ to PEG (100 mg C $L^{-1}$) -treated plants reverted the negative action of PEG on shoot growth with levels similar to those of SHA-treated plants (table I, 2). In line with previous discussion, this fact might derive from the interaction of SHA to cell pores that are not affected by PEG, since PEG concentration in solution in this experiment (0.01 %) is much lower than that normally used to generate a strong osmotic stress (6-10 %) (O'Donnell et al., 2013). This assessment is supported by the fact that when cucumber plants were treated with 10 % PEG, the application of SHA (100 mg C-SHA $L^{-1}$) was not able to reverse both the osmotic stress and shoot growth reduction caused by PEG. A fact that was reflected in the increase of ABA-leaf concentrations for both treatments 10 % PEG and (10 % PEG plus 100 mg C-SHA $L^{-1}$ ) (table I, 4). Further, this result also shows that proper $Lp_r$ function is essential for the SHA-mediated increase of shoot growth.

Regarding the mechanisms involved in the positive effect of 100 mg C $L^{-1}$ SHA on root water transport-related parameters, the results obtained suggest that the increase of ABA-root concentration caused by SHA might play a relevant role (figure I, 8). Numerous studies have shown the relevant role of root-ABA in regulating $Lp_r$ (Hose et al., 2000; Thompson et al., 2007; Tardieu et al., 2010; Sanchez-Romera et al., 2014) and the expression of genes encoding some families of plasma membrane aquaporin proteins as well (Beaudette et al., 2007; Mahdieh and Mostajeran, 2009). Assuming the limitations linked to all pharmacological approach due to the possibility of side effects of inhibitors and precursors on unwanted biochemical pathways, our experiments indicated that the SHA-mediated increase in $Lp_r$ is also ABA-dependent, since the application of Fld, a specific inhibitor of ABA biosynthesis in root and shoot, abolished SHA action on $Lp_r$. Interestingly, our experiments also showed that the

application of Fld on SHA-treated plants not only removed the SHA-mediated $Lp_r$ increase but also the shoot growth promoting action of SHA. In principle, in line with the results obtained in experiments using 10% of PEG, this finding indicates that the stimulation of $Lp_r$ is functionally involved in the enhancing effect of SHA on shoot growth, at least in cucumber. In fact, the SHA-mediated increase in both $Lp_r$ and shoot growth rate was correlated to an increase in $G_s$, which indicated higher metabolic activity in SHA-treated plants than in non-SHA treated plants.

Further, considering previous results showing that both the shoot promoting action of SHA and the SHA-mediated increase in ABA root concentration in cucumber were NO- and IAA-dependent (Mora et al., 2014a), the results obtained in the present study indicate that both NO and IAA regulate SHA effect on shoot growth in cucumber through ABA .

Several studies have shown that root-ABA regulates $Lp_r$ through pathways involving root plasma membrane aquaporin (PIPs) activity (Quintero et al., 1999; Beaudette et al., 2007; Vandeleur et al., 2014). It is therefore possible that the ABA-mediated effect of SHA on $Lp_r$ is expressed through the regulation of some *Cs*PIPs in roots. Our results showed that SHA application caused a significant up-regulation of the four *Cs*PIPs studied, with the time-course pattern of this action being different for each *Cs*PIP (figure I, 11). However, the effect of Fld on SHA-mediated *Cs*PIPs up-regulation was unclear. First of all, Fld application in control plants caused a significant up-regulation of *Cs*PIPs. This fact indicates that other ABA-independent regulatory pathways are probably activated in the root in order to prevent the evolvement of a potential water deficit stress resulting from ABA-functional loss. When SHA and Fld were applied together, two different effects were observed depending of the *Cs*PIP studied. On the one hand, an additive up-regulation for *Cs*PIP 1;2 and *Cs*PIP 2;5, and on the other hand an effect similar to Fld alone for *Cs*PIP 2;1 and, *Cs*PIP 2;4. In the first case the additive action of Fld and SHA is compatible with an effect of SHA on *Cs*PIP 1;2 and *Cs*PIP 2;5 gene expression through an ABA-independent pathway, while, in the second one, the similar action of Fld and SHA+Fld is compatible with and effect of SHA on *Cs*PIP 2;1 and *Cs*PIP 2;4 gene expression through an ABA-dependent pathway. Taking into account that this last case includes the most relevant *Cs*PIP in cucumber roots (*Cs*PIP 2;4) (Qian

et al., 2014), it is possible that the main ABA-mediated action of SHA on $Lp_r$ and water-root uptake involves the up-regulation of PIPs gene expression and activity.

In conclusion, the results obtained here show that: (i) The ability of SHA (a model sedimentary humic acid) to promote shoot growth in cucumber is mediated by an ABA-dependent enhancement of root hydraulic conductivity, water root uptake and root plasma membrane aquaporin gene expression, and (ii) SHA effects on shoot growth are probably integrated into a primary physicochemical interaction of SHA molecular complex with pores in cell walls at root surface, whose consequences on shoot growth (beneficial or detrimental) would depend of SHA concentration in the rhizosphere.

Interestingly, several studies suggested that effects like that of SHA in roots, involving changes in $Lp_r$ regulated by ABA root concentration, may result from some kind of transient stress, which in turn may have beneficial effects on plant growth and plant prevention from abiotic stresses (Wilkinson and Davies, 2002). In addition, data reported by Li et al. (2014) and Maurel et al. (2008) explained that PIPs can play an important role during early phase of water stress, by acting on root water transport activating $Lp_r$ though ABA biosynthesis in roots before a longer term inhibition. This framework is quite consistent with a mechanism of action for SHA on plant growth associated with the evolvement of a kind of stress resulting from the interaction of SHA with cell walls at root surface. The intensity or degree of stress caused by SHA would depend on SHA concentration in the rhizosphere: low SHA concentrations would cause a mild stress associated with beneficial effects on plant development while high SHA concentrations would cause strong stress associated with detrimental effects on plant development. Indeed, this hypothesis is in line with that proposed by Berbara and Garcia (2014) in order to explain their results showing an increase in root reactive-oxygen species (ROS) production caused by the application in rice of an HA obtained from vermicompost of vegetal residues. This action was in turn associated with the protection of rice plants against abiotic stresses (Berbara and Garcia, 2014). However, further studies are needed in order to elucidate the functional links between ABA-mediated effects of SHA on shoot growth and root water transport-related parameters, and HAs-mediated ROS production in roots. In addition, SHA mediated

response in plants regarding water relation parameters indicates that it is a typical response of early phase water stress in plants. Taking into account that the concentration applied to plants is in the beneficial range and final response of SHA treatment reaches the promotion of plant development, deeper studies are needed to elucidate if this beneficial transient stress disappeared in time or remains with sporadic activation and if this response is mediated by plant-SHA interaction that converges into a conformational change of SHA in solution and concomitant different stimuli for the plant.

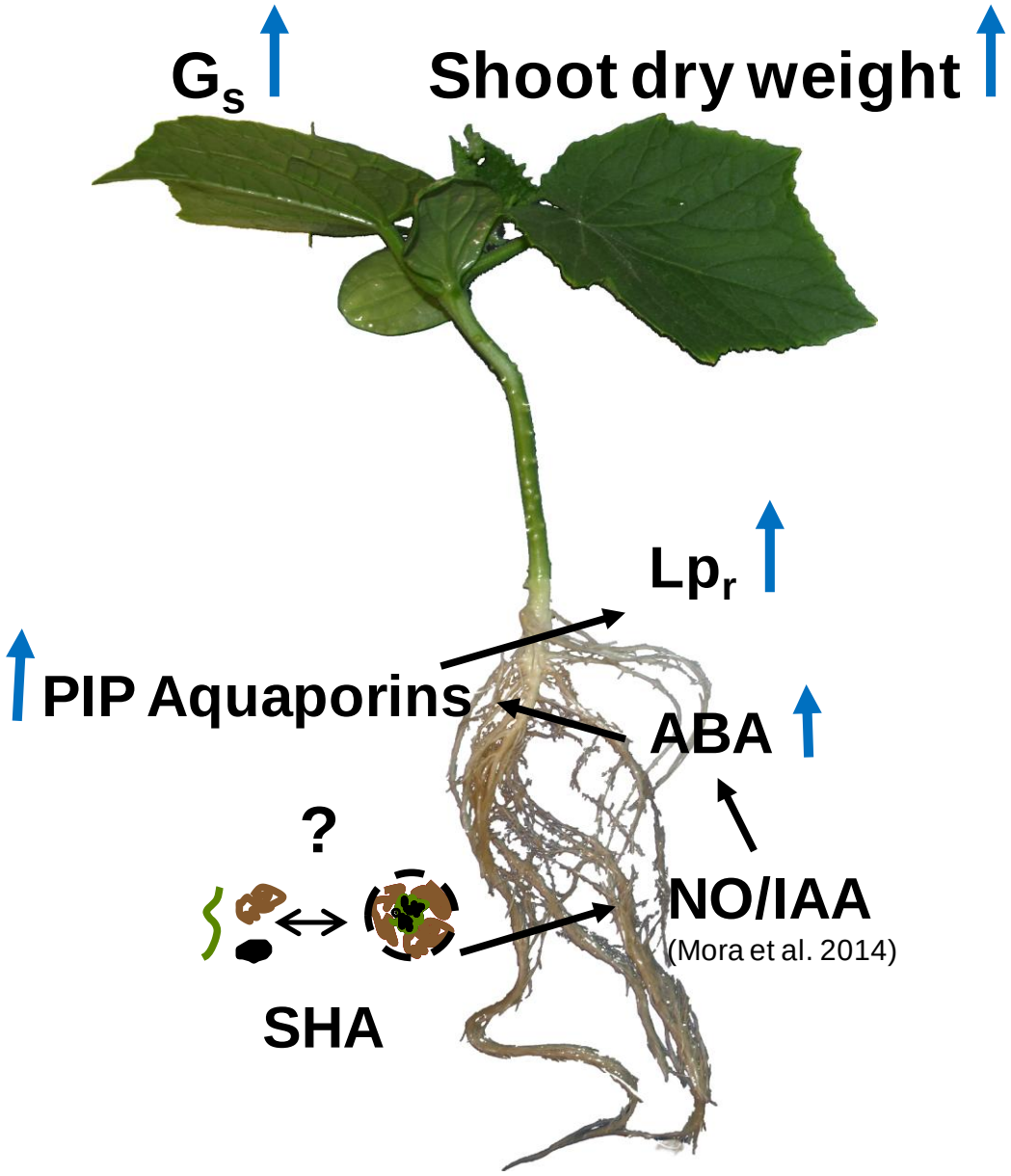


**Figure I, 13:** Diagram of the principal conclusions reached in chapter I.

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## CHAPTER II:

**Discriminating the relative role of root hydraulic conductivity ($Lp_r$) and root PM $H^+$-ATPase activity in the humic acid mediated enhancement of shoot and root growths in cucumber**

**Abstract**

HA ability to activate root PM $H^+$-ATPase and promote plant root development has been studied in detail (Canellas et al., 2002; Aguirre et al., 2009; Mora et al., 2010; Trevisan et al., 2010). Specifically, some studies reported the CHA ability to enhance the PM $H^+$-ATPase through mechanisms involving both IAA and nitric oxide (Zandonadi et al., 2010). With regard to the effects of humic acids on plant shoot, Mora et al., 2010 observed that the ability of a SHA to promote shoot growth was associated with root PM $H^+$-ATPase, linked to root-to-shoot translocation of Cks as well as root nitrate uptake and subsequent root-to-shoot translocation. Therefore, these events show the ability of HA mediated by $H^+$-ATPase activity to increase not only root growth but also shoot growth.

More recently, the ability of SHA to promote shoot growth through $Lp_r$ activation and mediated by root ABA and root plasma membrane aquaporin gene regulation has been described (Olaetxea et al., 2015).

Therefore, the crosstalk between both parameters is studied in this chapter, with a special focus placed on the involvement of $Lp_r$ or PM-$H^+$-ATPase activity in the growth of either of the two plant parts (shoot or root). In addition, root IAA biosynthesis is also studied, due to its ability to activate PM-$H^+$-ATPase. Results indicated that both parameters, $H^+$-ATPase activity and $Lp_r$, influenced SHA shoot growth promotion. However, in the case of root growth promotion, results showed that $H^+$-ATPase activity exerted a dependent pathway for the action of humic acid, although a potential role of $Lp_r$ cannot be ruled out.

**Introduction**

Many of the positive effects of humic acids (HA) on plants growing in soil have been described as a result of the improvement in the bioavailability of some macro- and micronutrients (Vaughan et al., 1985; Clapp et al., 2001; Chen et al., 2004). However, several studies showed that some humic acid-mediated effects on plant metabolism were difficult to explain as only being the result of an action of humic acids on plant mineral nutrition. In spite of the fact that many authors have investigated this type of effects of humic acids with different origins and in diverse plant models, all this information remains rather fragmentary since it has not been integrated into a more holistic mechanism.

With regard to the effects of humic acids on plant roots, studies carried out with humic acids extracted from composted materials (CHA) reported that the effects caused by this type of HA on lateral root proliferation and root architecture were partially expressed through auxin (indoleacetic acid, IAA)–dependent pathways (Muscolo et al., 2007; Schmidt et al., 2007). In line with these results, other studies reported that this effect is likely to be associated with the CHA ability to enhance the activity of root plasma membrane proton ATPase (PM-$H^+$ATPase) through mechanisms involving both IAA and nitric oxide (Zandonadi et al., 2010). This hypothesis referred to the "acid growth theory", which stated that cell elongation and root growth is regulated through mechanisms involving root PM-$H^+$ATPase activity, apoplastic acidification and auxin transport (Hager et al., 1991; Frias et al., 1996; Hager, 2003). In line with these results, studies employing sedimentary humic acids (SHA) reported that their application to cucumber roots caused significant increases in the root concentration of IAA, NO and ethylene as well as PM H+-ATPase activity (Mora et al., 2010, 2014a, 2014b). However, SHA-mediated increases in NO, IAA and ethylene did not explain all the SHA-effects on root development, such as dry matter production and secondary roots size diameter (Mora et al., 2014a, 2014b). In addition, other factors - besides those related to IAA, NO or ethylene -, have to be involved in the whole mechanism of action of SHA on root development. In this framework, the studies from the Berbara and Garcia group, which reported the relevant role of ROS in the regulation of the CHA enhancing effect on

secondary root proliferation and growth, suggest that ROS might be one of these new regulating factors (García et al., 2012).

With regard to the effects of humic acids on plant shoots, Mora et al. (2010) observed that the ability of SHA to promote shoot growth was associated with a significant increase in both the shoot-to-root translocation and the leaf-concentration of cytokinins, which were in turn linked to an increase in root $PMH^+$ - ATPase activity as well as root nitrate uptake and further root-to-shoot translocation. In line with this, some authors observed that the humic acid activation of root $PMH^+$ - ATPase was also linked to increases in nitrate uptake rates in roots of different plant species (Pinton et al., 1999; Nardi et al., 2002; Quaggiotti et al., 2004). These results also linked the effects of humic acids on shoot growth to their ability to enhance root PM $H^+$ - ATPase activity. Further studies showed that the promoting action of humic acids on shoot growth was expressed through auxin- and NO-dependent mechanisms (Mora et al., 2014a). Therefore, as in the case of the effects of humic acids on root development, all these results are also consistent with the existence of a relationship between the promoting action of humic substances on shoot growth and their ability to modify the root concentration of auxin and NO, and probably PM H+-ATPase activity (Root PM H+-ATPase-dependent pathway for humic acid action in plants) (Muscolo et al., 2007; Schmidt et al., 2007; Mora et al., 2010, 2014a; Canellas et al., 2002).

Recently, another study highlighted the relevant role of ABA-mediated increase in root hydraulic conductivity in the promoting action of SHA on shoot development in cucumber (Olaetxea et al., 2015) ($Lp_r$-dependent pathway for humic acid action in plants). In addition, this study also showed that this action of SHA was associated with a significant up regulation of root plasma membrane aquaporins (PIPs) as well as root water uptake rates and stomatal conductance in leaves (Olaetxea et al., 2015). Taking into account that the increase in root-ABA and in shoot growth as well, caused by SHA, were IAA- and NO-dependent (Mora et al., 2014a), it is possible that the $Lp_r$-dependent pathway and the PM-$H^+$-ATPase-dependent pathway are interconnected in the whole mechanism of action of humic acids on plant shoot and root development (Figure II, 1). However, none of the numerous published studies have provided experimental evidence that supports the idea that the humic acid-mediated enhancement of root

PM-$H^+$- ATPase activity plays, in fact, a crucial role in the promoting action of humic acids on plant shoot and/or root growth. In addition, the potential crosstalk between $Lp_r$ and root PM-$H^+$-ATPase in the action of humic acids in plants has not been addressed either.

In order to investigate these questions, we have studied the effects of an inhibitor of PM-$H^+$-ATPase activity and several molecular blockers of $Lp_r$ on the humic acid-mediated enhancement of both $Lp_r$ and PM-$H^+$-ATPase activity, as well as their consequences on shoot and root growths in cucumber.

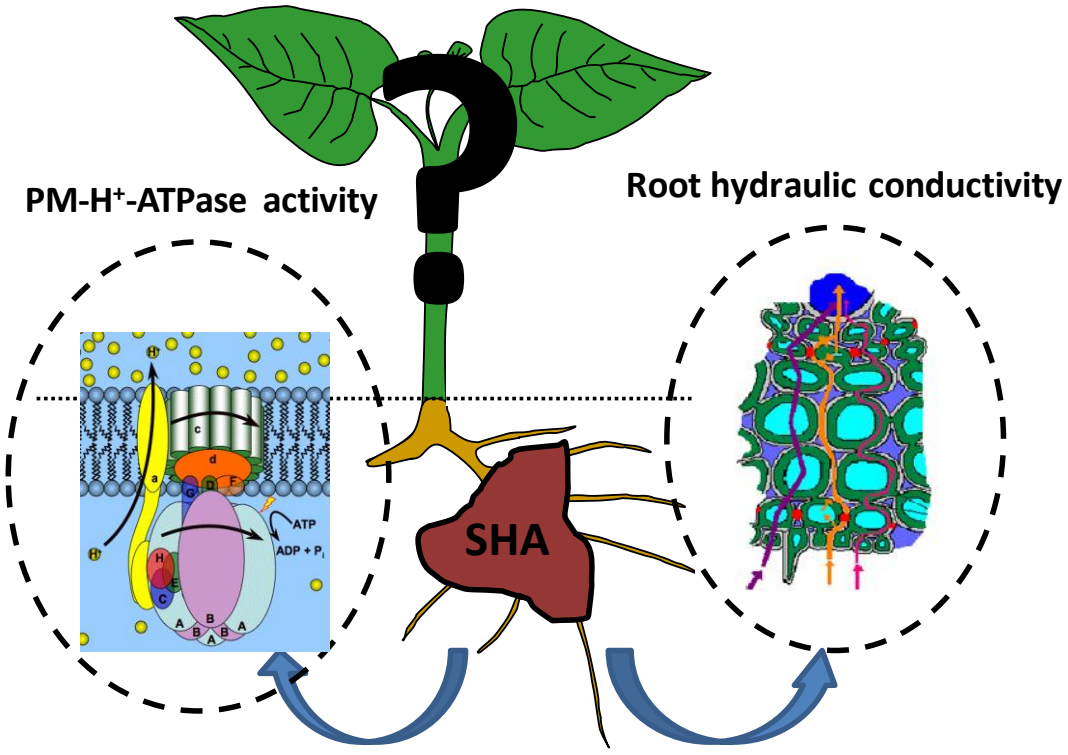



Figure II, 1: Hypothesis of study: involvement of SHA action on $Lp_r$ and/or PM-$H^+$ATPase in order to explain plant growth promotion (shared with Koning and Ross E., 1994)

## Material and Methods

### Extraction and purification of a leonardite humic acid (SHA)

The sample of HA obtained from leonardite (Danube basin) was used in the experiments. A specific amount of HA (100 g) was extracted, and purified using the IHSS methodology (http://www.humicsubstances.org/soilhafa.html) as described in (Aguirre et al., 2009). The concentration of the main phytoregulators in SHA composition was assessed employing (high performance liquid chromatography/ mass spectrometry) HPLC/MS as described in (Aguirre et al., 2009).

### Plant material and culture conditions

Seeds of cucumber (*Cucumis sativus* L. cv Ashley) were germinated in darkness with 1 mM of $CaSO_4$, on perlite and moistened filter paper in a seed germination chamber. One week after germination, plants were transferred to 8 L recipients in hydroponic solution. The nutrient solution used was: 0.63 mM $K_2SO_4$; 0.5 mM $KH_2PO_4$; 0.30 mM $MgSO_4$; 0.25 mM $KNO_3$; 0.05 mM KCl; 0.87 mM $Mg(NO_3)_2$ and 0.50 mM $Ca(NO_3)_2$; 40 µM $H_3BO_3$; 4 µM $MnSO_4$; 2 µM $CuSO_4$; 4 µM $ZnSO_4$ and 1.4 µM $Na_2MoO_4$. The nutrient solution contained 40 µM of iron as Fe-EDDHA chelate (80 % ortho-ortho isomer). No precipitation of Fe inorganic species was observed throughout the experiment. The pH of the nutrient solutions was held at 6.0 and did not change significantly during the experiment. All experiments were performed in a growth chamber at 25/21 ºC, 70-75% relative humidity and with 15/9 h day/night photoperiod (irradiance: 250 µmol $m^{-2}$ $s^{-1}$) and $CO_2$ content 400 ppm. Plants were grown in these conditions for 10 days before treatment application (see extended hydroponic culture conditions in annex).

### Experiments

Dhifferent experiments were undertaken in order to study the involvement of root PM $H^+$-ATPase and/or $Lp_r$ in the SHA-mediated effects on plant development.

1. Involvement of SHA on activation of $H^+$-ATPase and $Lp_r$ activities after application of $H^+$-ATPase inhibitor (N, N' dicyclohexyl-carbodiimide, DCC). In this experiment four plant treatments were applied: Control plants which

continued growing in nutrient solution for all the experiment. SHA treated plants (100 mg $L^{-1}$ of organic carbon added to the nutrient solution). DCC treated plants (5µM of DCC inhibitor for 30 minutes followed by transfer to the whole nutrient solution). DCC+SHA combined treatment (5µM of DCC inhibition treatment for 30 minutes followed by transfer to nutrient solution adding 100 mg $L^{-1}$ of organic carbon from SHA). The following physiological parameters were measured: shoot and root relative growth rate (SRGR) and (RRGR), $Lp_r$ and $H^+$-ATPase activity. This experiment was repeated three times.

2. Involvement of SHA on activation of $H^+$-ATPase and $Lp_r$ activities after application of $Lp_r$ inhibitors. Effects of SHA and $Lp_r$ blockers (PEG and PAA) treatments on $H^+$-ATPase and $Lp_r$ activities. In this experiment four treatments were studied: Control plants (grown in nutrient solution), SHA treated plants (application of 100 mg $L^{-1}$ of carbon of SHA to the nutrient solution) and two $Lp_r$ inhibitors (PEG (polyethylene glycol); nominal Mw of 20000Da and PAA (polyacrylic acid); nominal Mw of 32000Da) applied to the nutrient solution in 100 mg $L^{-1}$ carbon concentration. The choice of the molecular weight of PEG and PAA was done according to both the size of root cell-wall pores in cucumber (choosing a bigger hydrodynamic radius than plant pores) and its similarity to some SHA fractions apparent size. The following physiological parameters were measured: shoot and root relative growth rate (SRGR and RRGR) respectively, $Lp_r$ and $H^+$-ATPase activity and root IAA.

   A final treatment of PEG+SHA was also applied in order to study Influence of SHA on $H^+$-ATPase and $Lp_r$ activities after $Lp_r$ inhibition (the combined treatment using PEG was only applied because PEG is the most common $Lp_r$ inhibitor). SRGR RRGR and $Lp_r$ (values are presented in chapter I, figure I, 7) were measured.

All treatments involved a minimum of five replications. Harvests were conducted at the same time of the day to exclude diurnal variations, this meant 6 h after the start of the light period. Plants were harvested at 24, 48 and 72 h after the application of treatments. Some of the plant material were weighed and dried (60 °C) for Root and

Shoot Relative Growth Rate (SRGR and RRGR) determination, and some others were frozen in liquid nitrogen and stored at -80 °C for further analysis. Each experiment was repeated three times. Shoot and root relative growth determination was calculated as explained in Olaetxea et al. (2015) and in chapter I.

**Measurement of root hydraulic conductivity ($Lp_r$) in the absence of hydrostatic pressure gradients (free exudation method) ($Lp_r$)**

**Root exudates collection**: The root exudates were collected based on Gibbs et al. (1998a,b), Maurel et al. (2008) and Sanchez-Romera et al. (2014) works. The fundamental basis is explained in Olaetxea et al. (2015) and in chapter I.

Root exudate was finally collected with a glass Pasteur pipette. Collections were done continuously during the first 90 minutes of exudation at 24, 48 and 72 hours of treatment and kept in a previously weight 1.5 mL tube.

**Measurement of the osmotic pressure of exuded sap**: the osmolality of root exudates was measured using a freezing point depression osmometer (Osmomat 010 Gonotec, Germany) based on previous works of Bigot and Boucaud, (1998). Complete procedure is explained in Olaetxea et al. (2015).

Osmotic potential of solutions containing SHA, PEG or PAA was also evaluated and there were not significant differences between them.

**Analysis of IAA in roots**

The concentration of indole-3-acetic acid (IAA) was analyzed in root extracts using high performance liquid chromatography-electrospray-mass spectrometry (HPLC-ESI-MS/MS) as described in Jauregui et al. (2015). The extraction and purification of this hormone was carried out using the following the method described by Dobrev and Kaminek (2002) sligtly modified (Aguirre et al., 2009; Jauregui et al. 2015).

IAA was quantified by HPLC-ESI-MS/MS using an HPLC (2795 Alliance HT; Waters Co., Milford, MA, USA) coupled to a 3200 Q-TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface. A reverse-phase column (Synergi 4 mm Hydro-RP 80A, 150×2 mm; Phenomenex) was

used. The detection and quantification of the hormone was carried out using multiple reaction monitoring in the negative-ion mode, employing multilevel calibration curves with the internal standards as described in Jauregui et al. (2015).

**Analysis of Plasma Membrane $H^+$-ATPase activity in roots**

Plasma membrane (PM) vesicles were isolated from apical roots (3–5 cm) using a sucrose-gradient technique as previously described by Mora et al. (2010). Briefly, the root segments corresponding to the different treatments were cut separately and ground with a pestle in an ice-cold homogenization medium containing: 250 *mM* sucrose,10 % (v/v) glycerol, 10 *mM* glycerol-1 phosphate, 2 *mM* $MgSO_4$, 2 *mM* EDTA, 2 *mM* EGTA, 2 *mM* ATP, 2 *mM* DTT (dithiothreitol), 5.7 % (w/v) choline-iodine, 1 *mM* PMFS, 20 µg $mL^{-1}$ chymostatin, and 25 *mM* BTP (1,3-bis [TRIS (hydroxyl methyl) methyl aminopropane) buffered to pH 6.7 with MES. Approximately 2.5 mL $g^{-1}$ fresh weight of root tissues was used. The homogenates were filtered through four layers of cheese-cloth and subjected to 3 min of centrifugation at 13,000 g at 4 °C (Beckman Coulter Microfuge 22R Centrifuge). The pellets were discarded and the suspension was centrifuged for a further 25 min under the same conditions. The pellets were then recovered, gently resuspended in 400 mL homogenization medium, and loaded onto discontinuous density gradients made by layering 700 mL of 25 % (w/w) sucrose over 300 mL 38 % (v/v) sucrose cushion in 1.5 mL tubes. Both sucrose solutions were prepared in 5mM BTP MES, pH 7.4 and contained all the protectants present in the homogenization medium. The gradients were centrifuged for 1 h at 13,000 g, and the vesicles banding at the 25/38% interface were collected, diluted and prepared for enzyme activity measurements.

ATP-hydrolysing activity was measured by determining the release of inorganic phosphate with phosphate background correction.

**Statistical analysis**

Significant differences ($p < 0.05$) among treatments were calculated by using one-way analysis of variance (ANOVA) and the LSD Fisher *post hoc* test. All statistical test were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa USA). All the error

bars represent the standard error of each of the measurements. The mean values of the measured parameters were calculated with (n=5) independent samples. Only $Lp_r$ was calculated with n=7 independent samples.

**Results**

**Model validation: SHA application to cucumber plant roots enhanced both $H^+$-ATPase activity and $Lp_r$ as well as root and shoot growths**

The application of 100 mg $L^{-1}$ of C-SHA to cucumber plant roots caused significant increases in both $Lp_r$ and $H^+$-ATPase activity (table II, 1). These effects were associated with increases in the shoot- and root- relative growth rates (table II, 1). In relation to plant development, SHA treatment significantly increased shoot growth as well as root growth compared to control plants. (table II, 1).

Table II, 1: Effects of SHA treated plants on growth, $H^+$-ATPase activity and $Lp_r$.

| | RRGR[a] (mg $g^{-1}$ $d^{-1}$) | SRGR[a] (mg $g^{-1}$ $d^{-1}$) | $H^+$-ATPase activity[*] (μmolPi mg $pro^{-1}$ $min^{-1}$) | $Lp_r$[*] (g $H_2O$ $g^{-1}$ root DW $MPa^{-1}$ $h^{-1}$) |
|---|---|---|---|---|
| **C** | 229 b | 294 b | 0.44 b | 18.15 b |
| **SHA** | 336 a | 387 a | 0.53 a | 29.27 a |

[a] Shoot Relative growth rate between 24 and 72 hours of treatment

[*] Measured at 72 hours after treatments

**Inhibition of $H^+$-ATPase activity blocked the SHA promoting action on shoot and root growths as well as root hydraulic conductivity ($Lp_r$)**

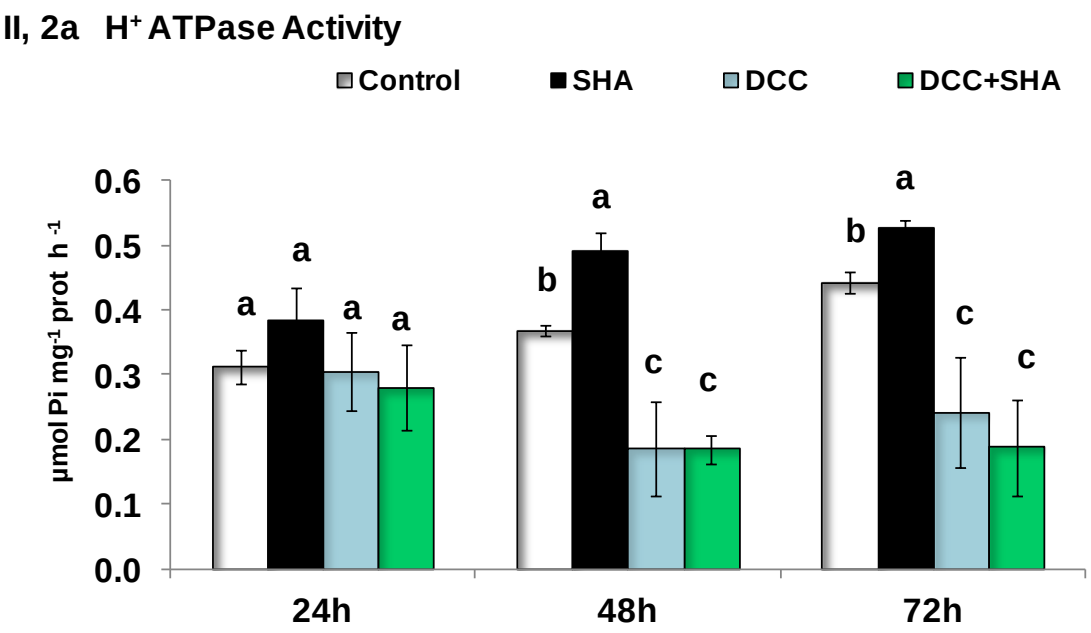
As expected the root application of DCC caused a significant decrease in the PM $H^+$-ATPase activity of control plants and removed the stimulation caused by SHA (Figure II,

2a). DCC application also significantly reduced SRGR and RRGR compared to control plants (table II, 2), and removed the increase caused by SHA (table II, 2).

Table II, 2: Shoot Relative Growth Rate (SRGR) and root Relative Growth Rate (RRGR) for SHA, DCC and DCC+SHA treated plants between 24 and 72 hours.

| | **SRGR (mg $g^{-1}$ $d^{-1}$)** | **RRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|---|
| **C** | 294 b | 229 b |
| **SHA** | 387 a | 336 b |
| **DCC** | 185 c | 57 c |
| **DCC+SHA** | 232 c | 86 c |

In relation to the influence of $H^+$-ATPase activity inhibition on $Lp_r$, DCC application significantly inhibited $Lp_r$ at 24, 48 and 72 h from the onset of treatments (figure II, 2b). Likewise, DCC fully removed the increase in $Lp_r$ caused by SHA after 24 and 48 h but only partially after 72 h (figure II, 2b).



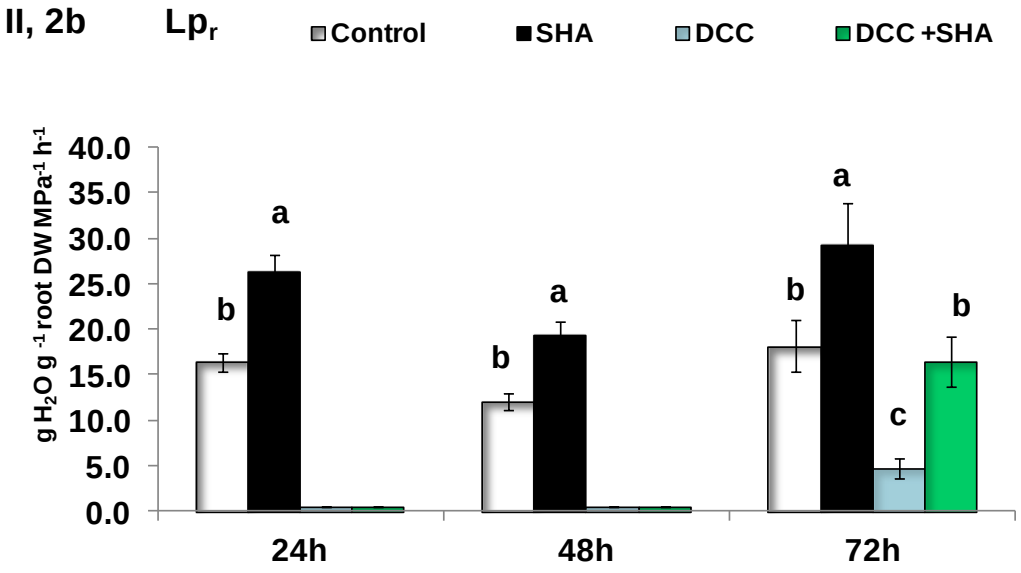




Figure II, 2: $H^+$-ATPase activity (II, 2a) and $Lp_r$ (II, 2b) for SHA, DCC and DCC+SHA treated plants between 24 and 72 hours of treatment. For $Lp_r$ measurements at 24 and 48 hours for DCC and DCC+SHA treatments totally blocked cell wall pores, there was not exudation.

**The application of PEG and PAA significantly reduced $Lp_r$ but increased both PM-$H^+$ ATPase activity and IAA root concentration**

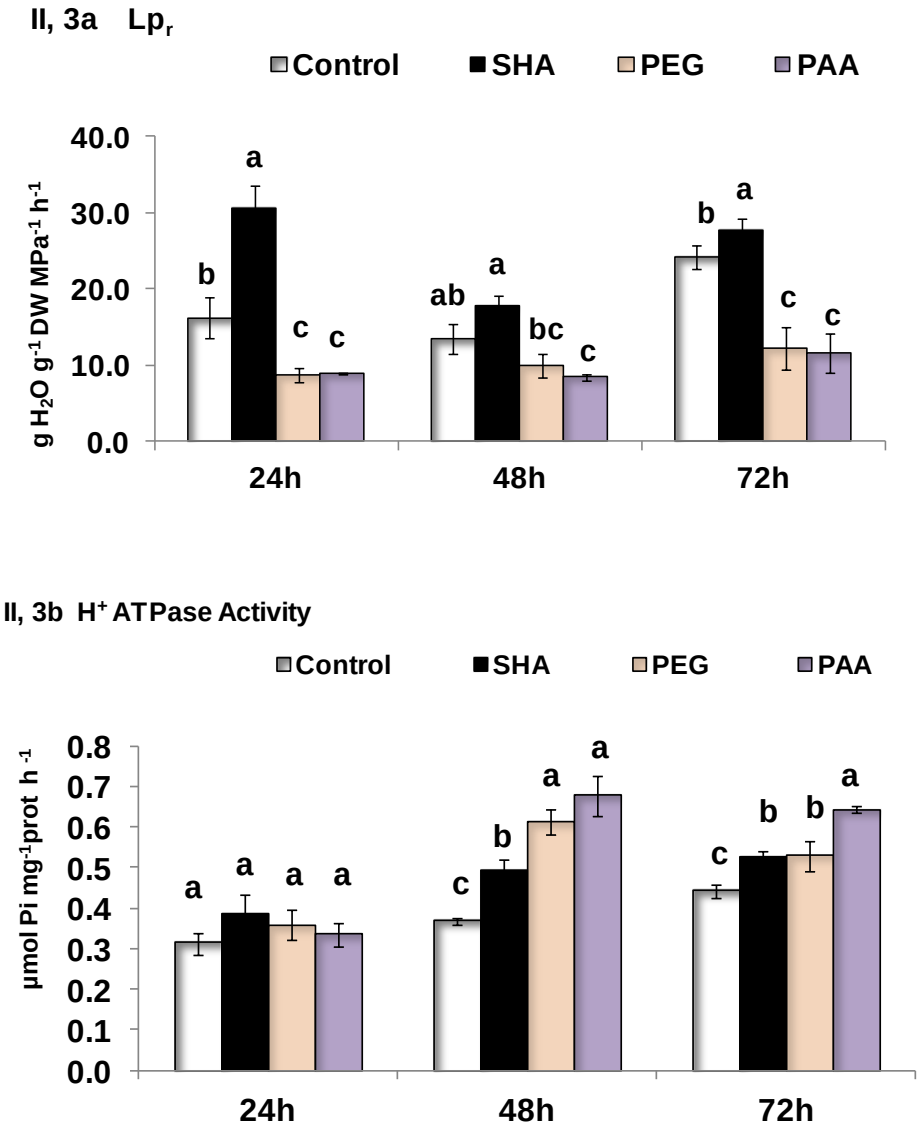



Figure II, 3: $Lp_r$ (figure II, 3a) and $H^+$-ATPase activity (figure II, 3b) for control, SHA, PEG and PAA treated plants measured at each harvest time between 24 and 72 hours.

As expected, the application of molecular blockers of $Lp_r$ (PEG and PAA) caused a significant inhibition of $Lp_r$ at all harvest times during the experiment (figure II, 3a). However, both PEG and PAA, were able to significantly increase $H^+$-ATPase activity at 48 and 72 hours compared to control plants even more than SHA (figure II, 3b). This effect was coupled with a significant prompt increase of root-IAA concentration (table

II, 5). However, both PEG and PAA significantly reduced SRGR compared with control and SHA-treated plants (table II, 3) while RRGR was not affected by these molecules compared with the control. When PEG and SHA were applied together, there was no significant differences on RRGR but as expected there was on SRGR (table II, 3).

Table II, 3: Shoot Relative Growth Rate (SRGR) and Root Relative Growth Rate (RRGR) for SHA, PEG and PAA treated plants between 24 and 72 hours.

| | **SRGR (mg $g^{-1}$ $d^{-1}$)** | **RRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|---|
| **C** | 294 b | 229 b |
| **SHA** | 387 a | 336 a |
| **PEG** | 204 c | 193 b |
| **PAA** | 216 c | 223 b |
| **PEG+SHA** | 364 a | 226 b |

Table II, 4: Root IAA concentration for SHA, PEG and PAA treated plants at 4 hours from the onset of treatment.

| **Root IAA (pmol $g^{-1}$ FW)** | **4h** |
|---|---|
| **C** | 63.12 b |
| **SHA** | 75.02 a |
| **PEG** | 71.64 a |
| **PAA** | 70.59 a |

**Discussion**

The presence of HA into root media –rhizosphere and fertirrigation root bulb for instance- affects not only plant root development and architecture but also shoot growth and fruit production (Sajid et al., 2012; Nithila et al., 2013). These effects result from a number of actions of HA in roots involving both soil-related factors (soil nutrient availability for plants, soil texture, porosity, oxygen and water exchange) and plant-related factors (transcriptional and post-transcriptional process and hormonal signaling pathways) (Aguirre et al., 2009; Trevisan et al., 2010; Canellas et al., 2002; Pinton et al. 1999; Quaggiotti et al. 2004; Mora et al. 2010, 2014a, 2014b). In this regard, one of the plant-related factors affected by HA is root PM $H^+$-ATPase activity at both transcriptional and post-transcriptional levels (gene expression, protein synthesis and enzyme activity) (Canellas et al., 2002; Quagiotti et al., 2004, Aguirre et al., 2009; Mora et al., 2010; Trevisan et al., 2010). Furthermore, these studies proposed that the HA-mediated enhancement of root PM $H^+$-ATPase activity is one of the key processes involved in the main mechanism of action of HA on plant performance and development regarding both root features (Nardi et al., 1991; Canellas et al., 2002;) and shoot growth (Mora et al., 2010). However, none of these studies have provided experimental evidence supporting this hypothesis. On the other hand, another study recently showed quite clearly that the promoting action of SHA on shoot growth in cucumber was linked to an ABA-dependent increase in $Lp_r$ (Olaetxea et al., 2015).

Our results here clearly show that the application of a biochemical inhibitor of root PM $H^+$-ATPase activity (DCC) removed the SHA-mediated increase in both RRGR and SRGR (Table II, 2). These results indicate that the enhancement in root PM $H^+$-ATPase activity caused by SHA appears to play a very relevant role in the root- and shoot-promoting actions of SHA, at least in cucumber. Furthermore, the application of DCC in control and SHA-treated plants also caused a significant decrease in $Lp_r$ values (figure II, 2b). In the case of SHA-treated plants, this effect of DCC was evident after 24 and 48 h but it declined after 72 h. Taking into account that the stimulation of $Lp_r$ played a crucial role in the effect of SHA on shoot growth (results presented in chapter I), these results indicate that, at least with regard to shoot growth, the removal of the enhancement caused by SHA might be the consequence of the DCC-effect on $Lp_r$. In this sense, there

are some studies describing the possible functional links between $Lp_r$ regulation and root PM $H^+$-ATPase activity, principally in plants subjected to abiotic stress such as cold (Lee et al., 2004) or salinity (López-Pérez et al., 2009). Lee et al (2004) reported that low temperatures caused significant decreases in root pressure (Pr), $Lp_r$, and root PM $H^+$-ATPase activity in cucumber. While the functional relationships between Pr and root PM $H^+$-ATPase activity were clearly established (Steudle, 1994; Ahn et al., 2000; Lee et al., 2004), those concerning $Lp_r$ and root PM $H^+$-ATPase activity were less clear. In fact, the regulation of aquaporin activity (PIPs), which in turn regulates $Lp_r$ by the activity of root PM $H^+$-ATPase, appears to be the only potential pathway involved among others that are also possibly working (Martínez-Ballesta et al., 2003). All these results could help explain our results. The inhibition of root PM $H^+$-ATPase activity by DCC caused a prompt reduction of $Lp_r$, even in the presence of SHA, which reflects the influence of root PM $H^+$-ATPase activity (active ion transport) on $Lp_r$ regulation.

The application of $Lp_r$ blockers (PEG and PAA), inhibited the $Lp_r$ activity (figure II, 3a) but interestingly it enhanced significantly $H^+$-ATPase activity after 48 hours of treatment (figure II, 3b). It needs to be noted that in these set of experiments, the block of $Lp_r$ with these treatments is of physical nature, because PEG and PAA are able to block cell wall pores (Asli and Neumann, 2010) and to stop the entrance of water. However, this physical block shows independency with $H^+$-ATPase activity at least at these conditions, because under these treatments $H^+$-ATPase activity was significantly enhanced (figure II, 3b). The $H^+$-ATPase activity was correlated with IAA-root biosynthesis, that was significantly enhanced at 4 hours (table II, 4). In relation to plant development, the application of PEG and PAA significantly decreased SRGR (table II, 3) wheres SHA enhanced it. The main difference between SHA and PEG or PAA is that whereas SHA increased both $Lp_r$ and root PM H+-ATPase activity, PEG and PAA increased root PM $H^+$-ATPase activity but decreased $Lp_r$, so these results suggest that $Lp_r$ is mainly involved in the promoting action of SHA on SRGR. In line with this reasoning, SHA was able to reverse the negative effect of PEG on shoot growth by recovering $Lp_r$ and showing significant differences in SRGR (when the treatment was applied together) (figure I, 7 and table II, 3).

With regard to root growth, neither PEG nor PAA affected RRGR (table II, 3). This result coincides with those obtained by Asli and Neumann (2010), who also observed that the root application of high concentrations of both PEG and HA inhibited shoot growth y but not root growth. However, although PEG and PAA did not decrease RRGR, they did not increase it either, as in the case of SHA. In this line, the application of combined treatment (PEG+SHA), neither was able to recover RRGR compared to both PEG or control plants (table II, 3). In this line, the inhibition of root PM $H^+$-ATPase activity by DCC also caused a drastic reduction in the RRGR in control plants (table II, 2). This result is consistent with the relevant role of the "acid growth theory" in plant root development (Hager et al. 1991; Frias et al. 1996). Likewise, DCC application to SHA-treated plants abolishes the increase in RRGR caused by SHA (table II, 2). In principle, although this effect supports a relevant role of the PM $H^+$-ATPase-dependent pathway in the action of humic acids on plant root development, a potential, also important, role of $Lp_r$-dependent pathway cannot be ruled out since DCC also reduced $Lp_r$ in control and (DCC+ SHA) treated plants (figure II, 2b). Our results indicate that the stimulation of root PM-$H^+$-ATPase activity probably plays a more relevant role in the mechanism of action of SHA on plant root growth than the increase of $Lp_r$ does. In principle, these results support the hypothesis proposed by several authors regarding the key role of PM-$H^+$-ATPase-dependent pathway in the effects of humic acids on plant roots (Canellas et al., 2002). However, the finding by previously published works (Mora et al., 2014a) that the inhibition of IAA-, NO- and ethylene-action, all of them directly related to PM $H^+$-ATPase activity regulation, did not affect root growth suggests that SHA might act on root growth through signaling pathways that are complementary to root PM $H^+$-ATPase- and $Lp_r$-dependent pathways. In this regard, the results reported by Berbara and Garcia group showing the high correlation between CHA-root ROS production and SHA-lateral root proliferation in rice (García et al., 2014) suggests that ROS-signaling pathways are most likely also involved in the signaling network associated with the action of HA on plant root features and growth.

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# CHAPTER III:

## Use of Humeomic methodology for exploring the structure-biological activity relationships of sedimentary humic acids

**Abstract**

The complexity and diversity of HS has not allowed to described in detail the structural basis of HS bioactivity in plants. In order to reduce molecular complexity of HS, different fractionation methods have been applied to HS. Fractionation is the method of subdividing HS into several fractions according to some property (Tan 2003). Recently and based on the supramolecular conformation theory of HS described by Piccolo (Piccolo, 2002), a novel chemical fractionation called "humeomics" has been described. It proposes a sequential isolation of molecules from humic supramolecular structure by breaking intermolecular bonds (Nebbioso and Piccolo, 2011). In agreement with the above mentioned, the aim of this chapter is first of all to subject SHA to humeomic fractionation method and to further characterize the chemical nature of the obtained fraction by different techniques (GC-MS, $^{13}$C NMR of FT-IR). Indeed, as an attempt to better understand the molecular conformation of SHA, a theoretical molecular model based on molecular mechanics will be proposed. Finally, the biological activity of the obtained fractions, specially plant growth promotion and some of the physiological parameters previously described (chapter I) will be evaluated and finally chemical structure-biological activity relations will be determined.

Results showed that after applying humeomic based fractionation process to SHA sample, 62.5% of the extracted material corresponded to the unbound fraction (ORG 1). In order to determine the physiological response of the obtained fractions, two fractions were studied: the ORG 1 fraction and the remained rest material (RES 1). The main structural difference between both samples was that RES 1 fraction showed higher aromatic character than ORG 1, that was more aliphatic.

RES 1 fraction increased $Lp_r$, root and shoot growth compared with control and bulk SHA treated plants. ORG 1 fraction instead, did not show significant differences on $Lp_r$ or shoot growth compared with control or SHA treated plants. The response in root of ORG 1 fraction was similar to SHA bulk treatment.

## Introduction

Researchers have made huge efforts studying the relationship between molecular structures of humic substances and biological activities. However, this issue remains rather unclear. Although structural properties such as macromolecular behavior, micelle-like character or supramolecular entity are evidences reached by different authors regarding HS chemistry, the complexity and diversity of HS has not allowed a detailed description of the structural basis of their bioactivity in plants (Zandonadi et al., 2013).

One of the first procedures by which humic substances can be conditioned for further studies is the extraction method. Most structure-activity concerning data refers to humic fractions obtained by the International Humic Substances Society (IHSS) proposed method, which is based on the solubility of HS in alkaline aqueous solution prepared with NaOH. Meantime, water extractable humic substances (WEHS) have also been described as plant growth promoters (Pinton et al., 1998 1999, Schmidt et al., 2007). In addition, several non-aqueous solvent extractions have been described as valid methods for obtaining active humic acids (Sun and Tomkinson, 2002)

Apart from the aforementioned extraction methods and in order to reduce the complexity and heterogeneity of humic or fulvic acids, fractionation processes have been widely applied to humic matter (Tan, 2003). The chemical and physical properties of humic substances addressed by each methodology configure the classification of the different fractionation methods. Chemical methods are generally based on differences in solubility in a specific solvent type, charge distribution or differences in adsorption. However, physical methods are based on differences in density, particle size and molecular weights (Tan, 2003).

In the framework of the supramolecular nature of humic substances by Piccolo and co-workers (Piccolo, 2002; Nebbioso and Piccolo, 2011), a novel chemical fractionation has recently been proposed. This new approach has been given the name “humeomics”, and it involves a sequential isolation of molecular families from humic

supramolecular structures by breaking specific intermolecular and intramolecular chemical bonds (Nebbioso and Piccolo, 2011). The different fractions obtained are subsequently extensively characterized by using common techniques for structural elucidation such as carbon and proton NMR, FTIR, size exclusion chromatography, Gas-Mass spectrometry and Liquid-Mass spectrometry; among others (Saiz-Jiménez 1995; Amir et al., 2003; Ait Baddi et al., 2004a, b; Baigorri et al., 2007; Fuentes et al., 2007).

Within this framework, the aim of this study is to investigate the structure-activity relationships using the humeomic fractionation approach in cucumber (*Cucumis sativus* L. cv Ashley). To this aim, we have isolated and characterized the different fractions resulting from the application of humeomic methodology to SHA. Then, we selected the two fractions resulting from the application of the first step humeomics to investigate their ability to promote root and shoot growth in comparison with SHA. This selection was made because the first step of humeomics involves the disruption of intra- and intermolecular attraction forces with low covalent character such as hydrogen bonds or van der walls forces, a process that may occur in the rhizosphere due to the interaction of SHA with root surface and/or root exudates (Canellas et al., 2008). Finally, a tentative SHA molecular model was also used to describe the supramolecular conformation of SHA. Evidently, this model is only a proxy that has to be further completed from humeomics, since it was merely based on elemental composition, $^{13}$-C-NMR functional distribution and DLS size distribution.

This work has been carried out in collaboration with Prof Alessandro Piccolo and coworkers in Università degli Studi di Napoli, Federico II.

## Material and methods

### Extraction, purification and modeling of a leonardite humic acid (SHA)

The sample of SHA obtained from leonardite (Danube basin) was used in the experiments. A specific amount of HA (100 g) was extracted, and purified using the IHSS methodology (http://www.humicsubstances.org/soilhafa.html) as described in (Aguirre et al., 2009). The concentration of the main phytoregulators in SHA

composition was assessed employing high performance liquid chromatography/ mass spectrometry (HPLC/MS) as described in Aguirre et al., 2009. SHA was characterized using $^{13}C$ nuclear magnetic resonance ($^{13}C$ NMR) (figure III, 2), elemental analysis (table III, 1) and size distribution ($R_h$) by Dynamic Light Scattering (DLS) (figure III, 3).

SHA conformation (primary, secondary and tertiary structures) was theoretically modeled based on data from Elemental Analysis, $^{13}C$ NMR and DLS. Its geometry was optimized by using Molecular Mechanics (AMBER method) (Hyperchem 8.0) (figure III, 5)

*Chemical fractionation and characterizations of SHA using humeomics (humeomic fractionation and further fraction characterization using GC-MS and NMR spectroscopy) were carried out in the University of Naples following the procedures of Nebbioso and Piccolo (2011).*

A previously extracted and purified sample of SHA was subjected to humeomic chemical fractionation. First of all, the original SHA (solved in NaOH and freeze dried) was suspended in HCl (pH 1) and dried. The operational fractionation process involved the following steps:

(i) Unbound fraction: The unbound fraction called ORG 1 throughout the document, was extracted by stirring 0.5 g of SHA (named RES 0) in 300 mL of a 2:1 v/v dichloromethane (DCM):methanol (MeOH) solution for 24 hours and at room temperature. The supernatant was separated by centrifugation (15 min, 15000 rpm) and filtered using a Whatman GFC (47mm) filter under vacuum. Finally, the obtained supernatant (named RES 1) was air-dried before the next step.

(ii) Weakly bound ester fraction: Previously separated residue (RES 1) was suspended in Teflon tubes with 12% of $BF_3$-MeOH using a ratio of 0.075 mL of solution per milligram of RES 1 and kept it in an oven at 85ºC overnight. The supernatants were centrifuged (15 min, 7000 rpm) and the residual $BF_3$ was quenched with water and the solution was extracted three times with a total amount of 80 mL (50:50 v/v) of chloroform/water mixture. In this step we obtained two phases: the organic phase called ORG 2, which was

filtered through Whatman GFC (47mm) and dried with anhydrous $Na_2SO_4$, and the aqueous phase named AQU 2 that was dialyzed using 1000 Da cutoff membranes against distilled water. The remaining solid residue (named RES 2) was air-dried and used for the next step.

(iii) Strongly bound ester fractions: RES 2 was suspended in 1M KOH-MeOH solution with a ratio of 0.25 mL per mg of RES 2 and refluxed for 2 h at 70ºC under $N_2$ atmosphere. After cooling, the reaction mixture was washed with MeOH and centrifuged (4500 rpm, 10 min). Before the isolation of each of the fractions, the pH of supernatant was adjusted to 2 with HCl 37%, and then a liquid-liquid extraction was applied with 100 mL DCM/water for three times. The organic-soluble fraction (named ORG 3) and the hydrosoluble fraction (named AQU 3) were purified as described in the previous step. The remaining solid residue (named RES 3) was air-dried and subjected to the next step.

(iv) Strongly bound ether fraction and final residue: RES 3 was treated with an aqueous solution of HI (0.25 mL of HI 47% per milligram of residue) and stirred for 48 h at 75ºC under $N_2$ atmosphere. After cooling, the reaction mixture was centrifuged (10 min, 3000 rpm) and filtered through Whatman GFC 47 mm. In this step we obtained the final solid residue (named RES 4) that was dialyzed, and the organic phase (named ORG 4) that was neutralized with $NaHCO_3$ and treated with $Na_2S_2O3$. In our study we did not obtain the ORG 4 fraction.

(v) The solvent of all the organic fractions was evaporated and dried with $N_2$ until its total removal.

All fractions were weighed before any analytical determination in order to evaluate the yield recovery associated with the fractionation process.

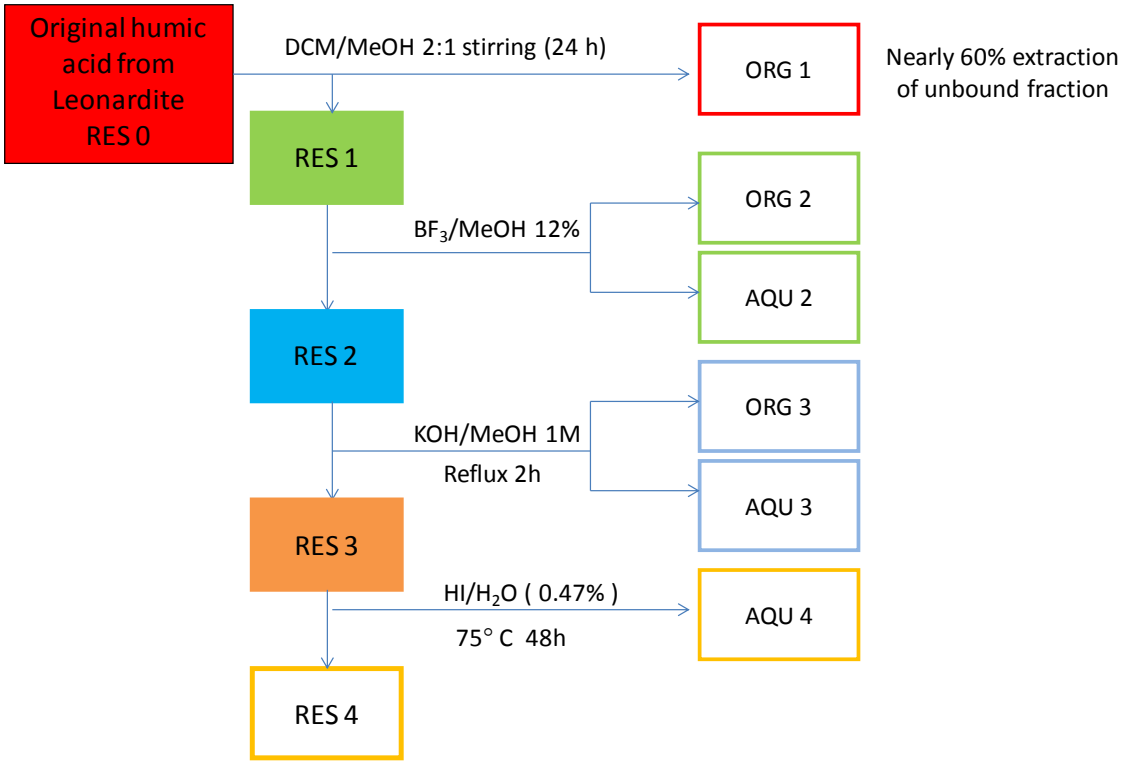



Fig III. 1. Fractionation process based on humeomics applied to leonardite extracted SHA. Diagram of the applied extraction or reaction procedures, and the obtained fractions.

**Gas Chromatography-Mass spectrometry (GC-MS)**

Organic-soluble fractions were analyzed using GC-MS previous derivatization with adequated agents. First samples wre derivatized by refluxing 1mg of dry sample with 0.5 mL of MeOH and excess of acetyl chloride for 30 min at 70°C. After the reaction, samples were cooled and dried under $N_2$. For the silylation step, we added 100 µL of pyridin on the vial and 100 µL of the silylation reagent (N,N-bis[trimethylsilyl]trifluoracetamide/1% trimethylchlorosilane, BSTFA) leaving the mixture in reaction for 35 min at 60°C. Then the reaction mixture was cooled down and put in vials for auto sampler. 10 µL of nonadecanoic acid were added as an internal standard. GC-MS analyses were conductedwith a Perkin-Elmer Autosystem XL gas chromatograph, equipped with a Perkin-Elmer turbomass gold mass spectrometer. The injector was set at 250°C, and a low-polarity phase fused-silica capillary column

(Restek Rtx-5MS, 30 m length x 0.25 mm I.D x 0.25 µm film thickness) was used under a helium flow rate of 1.6 mL min $^{-1}$. The oven temperature increased from 100 to 300°C at 4°C min$^{-1}$ and kept constant for 55 min. The mass spectrometer operated in full scan mode in the *m/z* 50-600 range, with EI energy of 70 eV and cycle time of 1.0 s.

Compound identification was supported by NIST-MS library mass spectra. For the integration procedure and compound identification, peak areas ≤5% of the mayor peak were not integrated. S/N ratio ≥ 10 was also respected. Three experimental independent fractions were analyzed for each of the fractions but only two spectra of each fraction are presented in the document. Quantitative measurements were not carried out because external calibration curves of known standards were not obtained. Qualitative identification of sample components was carried out.

**NMR spectroscopy**

Solid-state CPMAS$^{13}$C-NMR (crosspolarization magic angle spinning$^{13}$C-nuclear magnetic resonance) spectra were acquired with a Bruker AV 300 instrument equipped with a 4 mm wide bore MAS probe. Samples were fitted in 4 mm Zirconia rotors with Kel-F caps and spun at 13 000 ± 2 Hz. A recycle time of 1.0 s and an acquisition time of 20 ms were used, and 1510 points were acquired for each spectrum. The scan number ranged between 500 and 700. Variable spin lock (VSL) experiments were acquired with a 3 s recycle delay, 30 ms acquiring time, 2200 scans, and a VSL RAMP sequence with 1 ms contact time. An average spin lock frequency of 60 MHz was applied during the ramped cross-polarization time. Spin lock was varied in intervals from 0.01 to 7.50 ms in 21 increments. Dipolar dephasing (DD) experiments were set with a 15 µs delay and 15 000 scans. Data were processed with Mestre-C software 4.9.9.9, and all FID spectra were transformed with 100 Hz line broadening exponential type filter function and 2k zero filling.

**Elemental analysis**

Elemental analysis of the bulk HA sample was measured by a LECO CHN-2000 and the oxygen was calculated by difference. 0.2g of weight sample was prepared for each of the measurements which were repeated twice.

**FT-IR spectroscopy**

Infrared spectra of both ORG1 and RES1 were recorded with a Nicolet Magna-IR 550 spectrometer over the 4000–400 $cm^{-1}$ range.

**Plant material and culture conditions**

Seeds of cucumber (*Cucumis sativus* L. cv Ashley) were germinated in water with 1 mM of CaSO4, in darkness, on perlite and moistened filter paper in a seed germination chamber. One week after germination, plants were transferred to 8 L recipients in hydroponic solution. The nutrient solution used was: 0.63 mM $K_2SO_4$; 0.5 mM $KH_2PO_4$; 0.5 mM $CaSO_4$; 0.30 mM $MgSO_4$; 0.25 mM $KNO_3$; 0.05 mM KCl and 0.87 mM $Mg(NO_3)_2$; 40 µM $H_3BO_3$; 4 µM $MnSO_4$; 2 µM $CuSO_4$; 4 µM $ZnSO_4$ and 1.4 µM $Na_2MoO_4$. The nutrient solution contained 40 µM of iron as Fe-EDDHA chelate (80 % ortho-ortho isomer). No precipitation of Fe inorganic species was observed throughout the experiment. The pH of the nutrient solutions was held at 6.0 and did not change significantly during the experiment. All experiments were performed in a growth chamber at 25/21 °C, 70-75% relative humidity and with 15/9 h day/night photoperiod (irradiance: 250 µmol $m^{-2}$ $s^{-1}$).

As explained before, all treatments involved a minimum of five replications. Harvests were conducted at the same time of the day to exclude diurnal variations, this meant 6 h after the start of the light period. Plants were harvested at 24 and 72 h after the application of treatments. Plant material was weighed and dried (60°C) for Shoot and Root Relative Growth Rate (SRGR and RRGR) and $Lp_r$ determination was also carried out (explained in Olaetxea et al. 2015).

Three treatments were considered in these experiments: bulk or whole SHA, and fractions resulting from the application of the first humeomic step: ORG 1 and RES 1.

**Measurement of root hydraulic conductivity ($Lp_r$) in the absence of hydrostatic pressure gradients (free exudation method)**

Root exudates collection: the root exudates were collected based on Gibbs et al. 1998a and 1998b), Maurel et al. (2008) and Sanchez-Romera et al. (2014) works. The fundamental basis is explained in Olaetxea et al. (2015).

Root exudates were finally collected with a glass Pasteur pipette. Collections were done continuously during the first 90 minutes of exudation at 24 and 72 hours of treatment and kept in a previously weight 1.5 mL tube.

Measurement of the osmotic pressure of exuded sap: the osmolality of root exudates was measured using a freezing point depression osmometer (Osmomat 010 Gonotec, Germany) based on previous works of (Bigot and Boucaud, 1998). Complete procedure is explained in Olaetxea et al., 2015

Osmotic potential of solutions containing either bulk SHA, ORG 1 or RES 1 was also evaluated and there were no significant differences between them.

**Statistical analysis**

Significant differences ($p < 0.05$) among treatments were calculated by using one-way analysis of variance (ANOVA) and the LSD Fisher *post hoc* test. All statistical test were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa USA). All the error bars represent the standard error of each of the measurements. The mean values of the biological parameters were calculated with (n=5) independent samples. Only $Lp_r$ was calculated with n=7 independent samples.

**Results**

**Characterization of bulk SHA**

The results obtained from Elemental analysis, $^{13}C$ Nuclear Magnetic Resonance ($^{13}C$-NMR) and DLS- molecular size distribution are presented in tables (table III, 1 figure III,

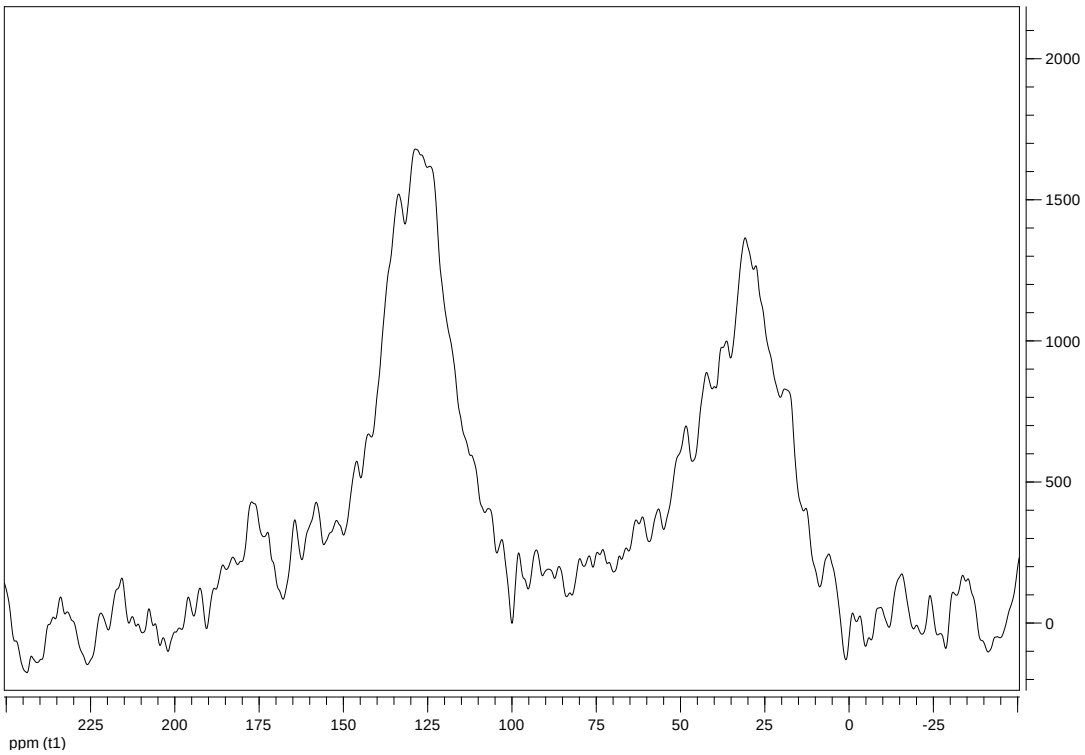
2). SHA presented 48.2% C, 47.7% O, 2.99% H quantity and around of 1% for both N and S (0.98 and 0.14 % respectively).

In relation to the aromatic and aliphatic character of SHA, $^{13}$Carbon Nuclear Magnetic Resonance ($^{13}$C-NMR) spectra shows that SHA bulk sample presents higher proportion of aromatic carbons (38.6%) compared with aliphatic carbons (31.4%). The rest of functional groups corresponded to O-alkyl C (14.4%), carboxylic C (11.6%) and carbonylic C (3.90%). Finally, DLS analysis of SHA in solution after filtration through 0.1μ m filters. DLS spectrum shows that there are two major populations of particles, corresponding to 5.11 and 14.1 nm of medium hydrodynamic radii (figure III, 3).

In order to propose a tentative molecular model for SHA, a primary structure was prepared using the information deriving from elemental composition and $^{13}$C-NMR (aromatic C, aliphatic C and functional group distribution) as well as some humic acid models proposed in other studies (Albers and Hansen, 2010; Schaumann and Thiele-Bruhn, 2011). This primary structure was modeled using molecular mechanics (Amber force field) (Figure III, 4). As a function of the size distribution obtained from DLS a secondary and tertiary supramolecular structure were also proposed based on the interactions of SHA sub-units through weak attraction forces like hydrogen bonds and van der walls forces (figure III, 5; figure III, 6 and figure III, 7). In figure III, 5 the domain formed by 3 sub-units of the primary structure are arranged and modeled. The size of the domain was theoretically fixed in 5.64 nm which could be correlated with DLS measurements (figure III, 3). In figure III, 6 a tertiary supramolecular structure was modeled. In this model, 6 sub-units were arranged and the size of the particle was theoretically fixed in 10.90 nm.

Table III, 1: Elemental analysis for SHA sample. Oxygen is calculated by difference.

| | **%C** | **%H** | **%N** | **%S** | **%O*** |
|---|---|---|---|---|---|
| SHA | 48.2 | 2.99 | 0.98 | 0.14 | 47.4 |




| Region (ppm) | Alkyl C 0-45 | O-Alkyl C 45-110 | Aromatic C (Phenolic C) 110-160 (140-160) | Carboxilyc C 160-190 | Carbonylic C 190-230 |
|---|---|---|---|---|---|
| SHA | 31.4 | 14.4 | 38.6 (14.7) | 11.6 | 3.90 |

Figure III, 2: $^{13}$Carbon Nuclear Magnetic Resonance ($^{13}$C-NMR) spectra for SHA sample and the corresponding percentage for each of the regions.

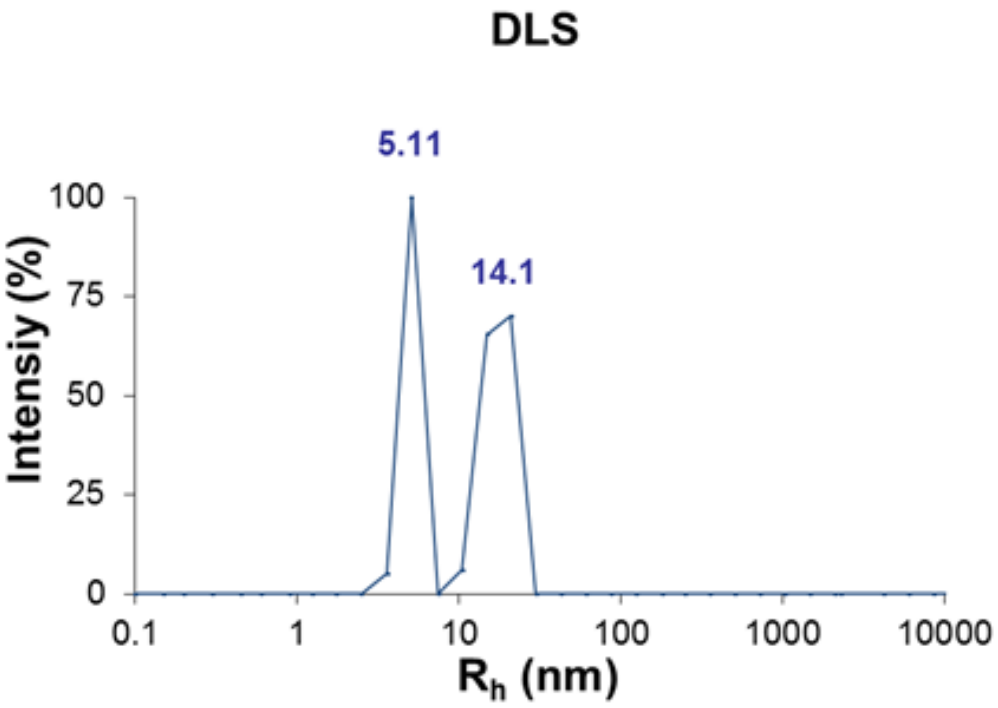



Figure III, 3: DLS spectrum for SHA humic sample.

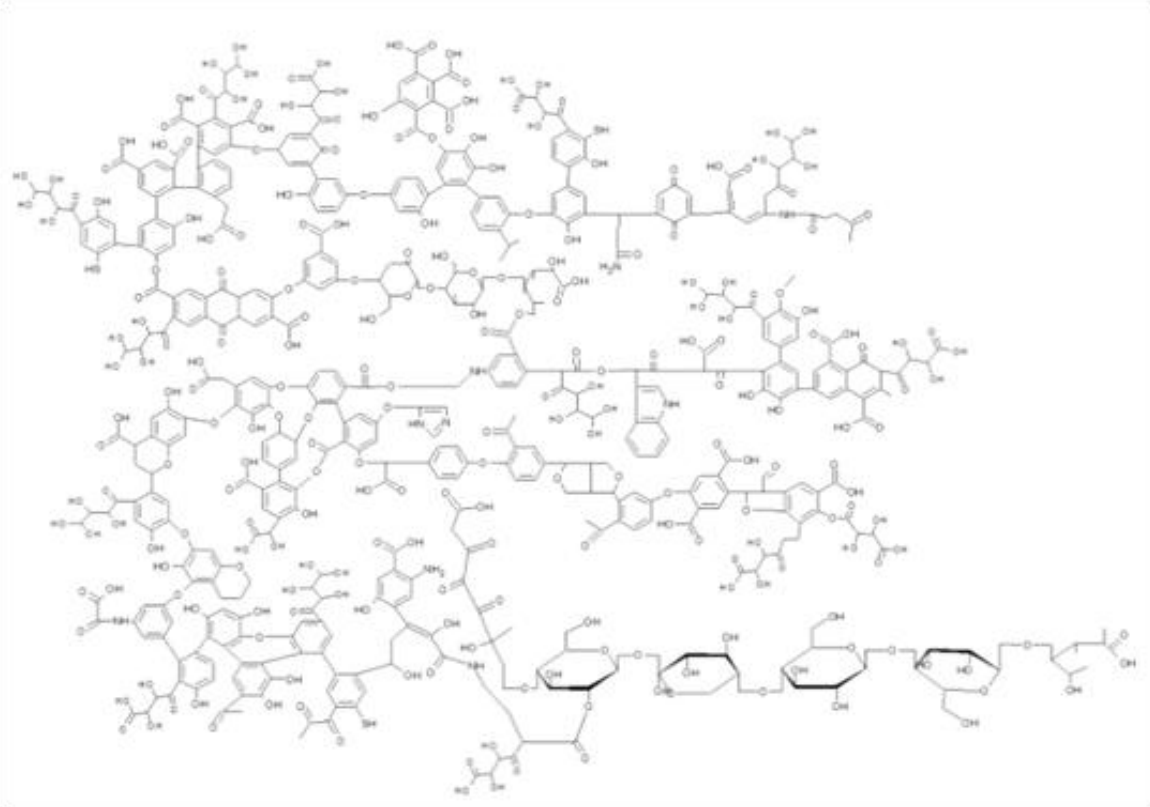


Figure III, 4: Primary structure for SHA model based on elemental composition and $^{13}$C-NMR (aromatic C, aliphatic C and functional group distribution).

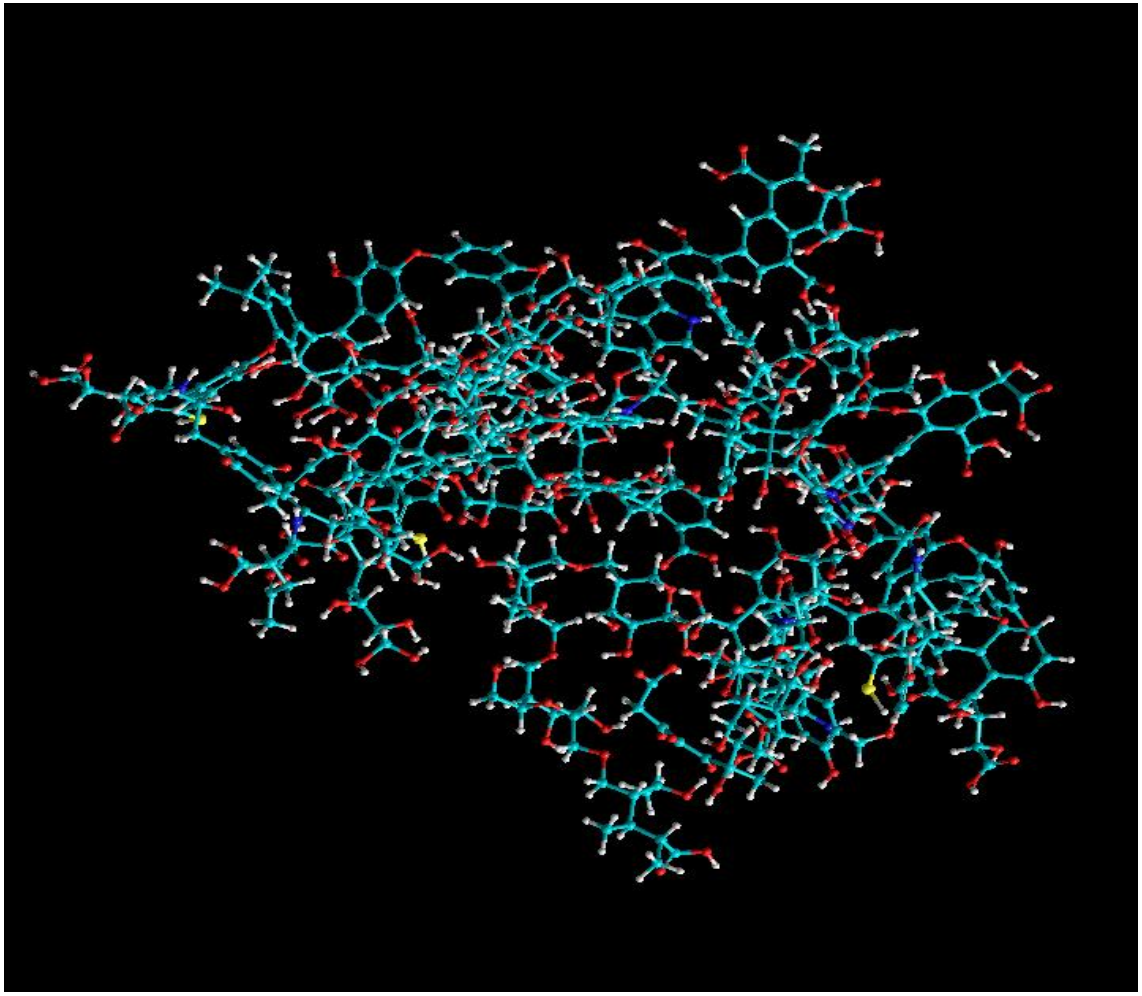


Figure III, 5: Modeling of the primary structure using molecular mechanics (Amber force field).

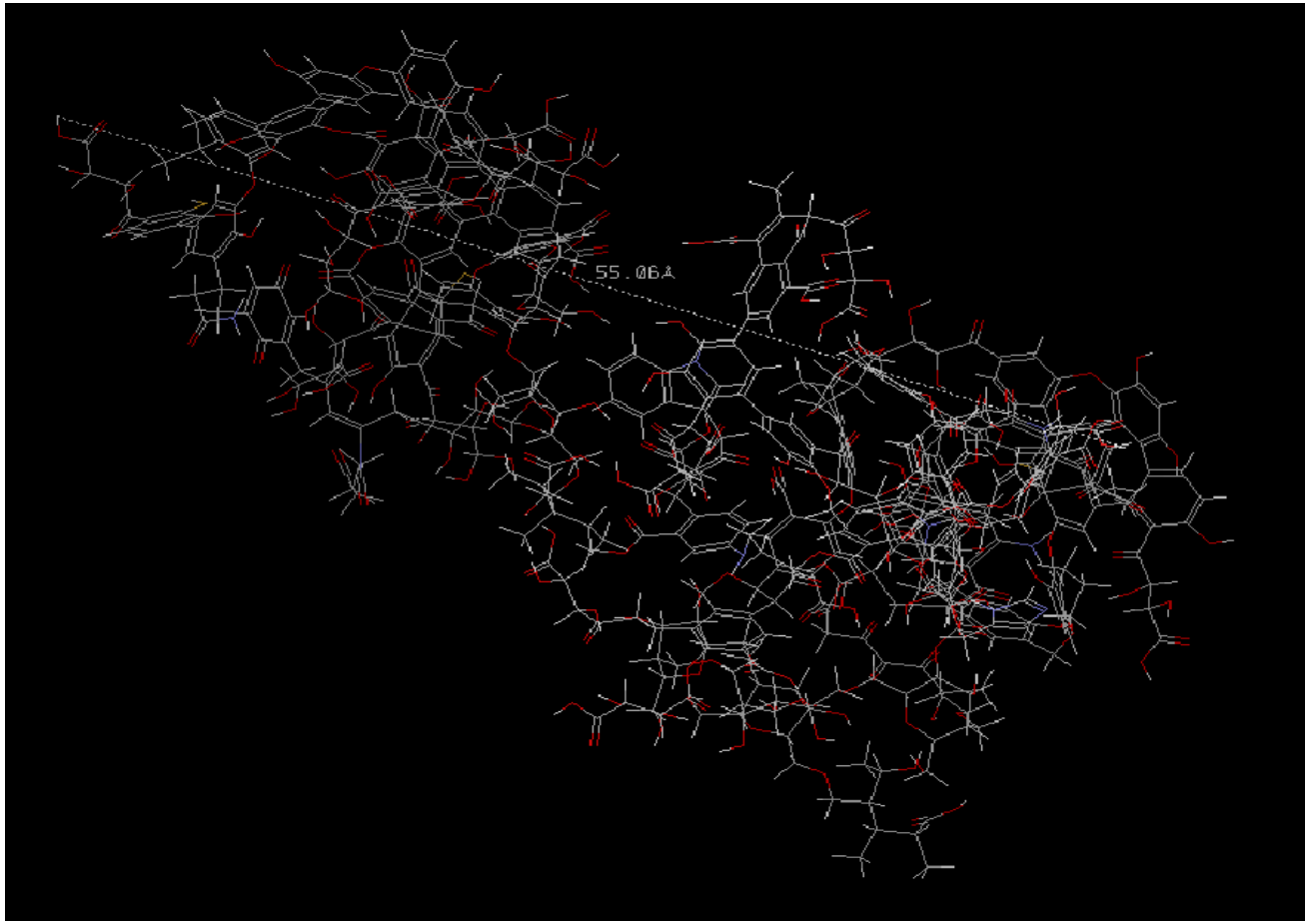



Figure III, 6: Theoretical particle size measurements of three sub-units coexisting in the secondary structure of the model SHA resulting from the primary structure and DLS study. The size particle was theoretically fixed in 5.64 nm.

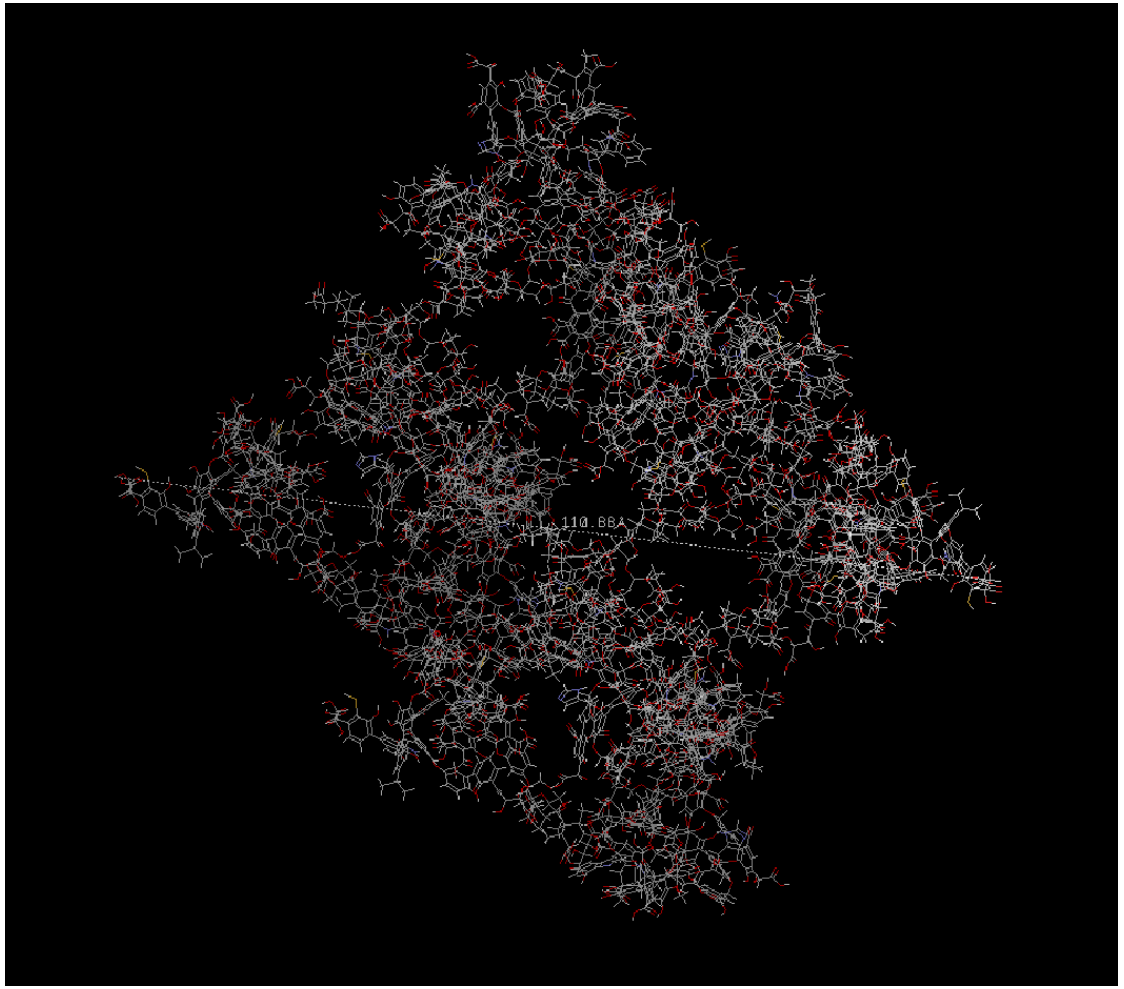



Figure III, 7: Theoretical particle size measurements of the domain of six sub-units arranged through weak attraction forces like hydrogen bonds and Van der Walls forces. The size of the particle was fixed in 10.90 nm.

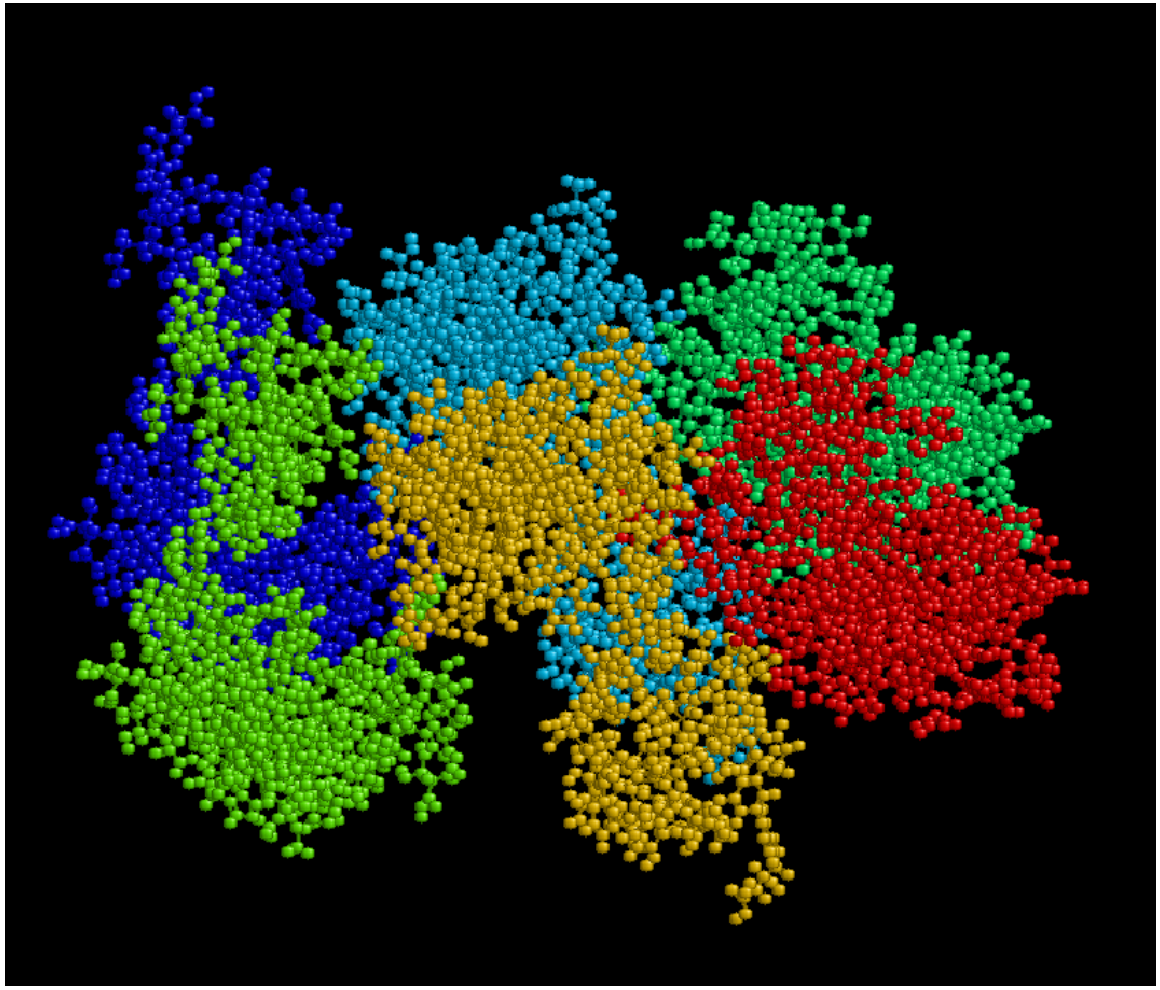


Figure III, 8: Theoretical molecular secondary structure proposed by molecular mechanics for SHA. In this figure the six sub-units are represented in colors

**Gravimetric characterization of the fractions obtained by the humeomic application to SHA**

Table III. 2: Percentage in mass for each of the samples extracted by humeomic-based sequential fractionation.

| Sample | Percentage (%) |
|---|---|
| RES 0 | 100 |
| ORG 1 | 62.5 (±4.25) |
| ORG 2 | 12.1 (±2.46) |
| ORG 3 | 13.6 (±4.31) |
| AQU 2 | 0.17 (±0.02) |
| AQU 3 | 0.49 (±0.56) |
| AQU 4 | 1.03 (±0.15) |
| RES | 9.97 (±1.49) |

Percentage of sample calculated against the original sample (RES 0). Standard deviation in parentheses.

The main fraction obtained with 62.5% in weight of the total amount of the initial sample (RES 0), was ORG 1. The following sequential fractionation yielded quite large amounts for ORG 2 and ORG 3 compared to the AQU fractions that yielded too small amounts, reaching scarcely 1% of the amount of the initial sample. The whole fractionation process yielded a final refractory and stable residue (RES 4) of 9.97% of the initial material (Table III, 2).

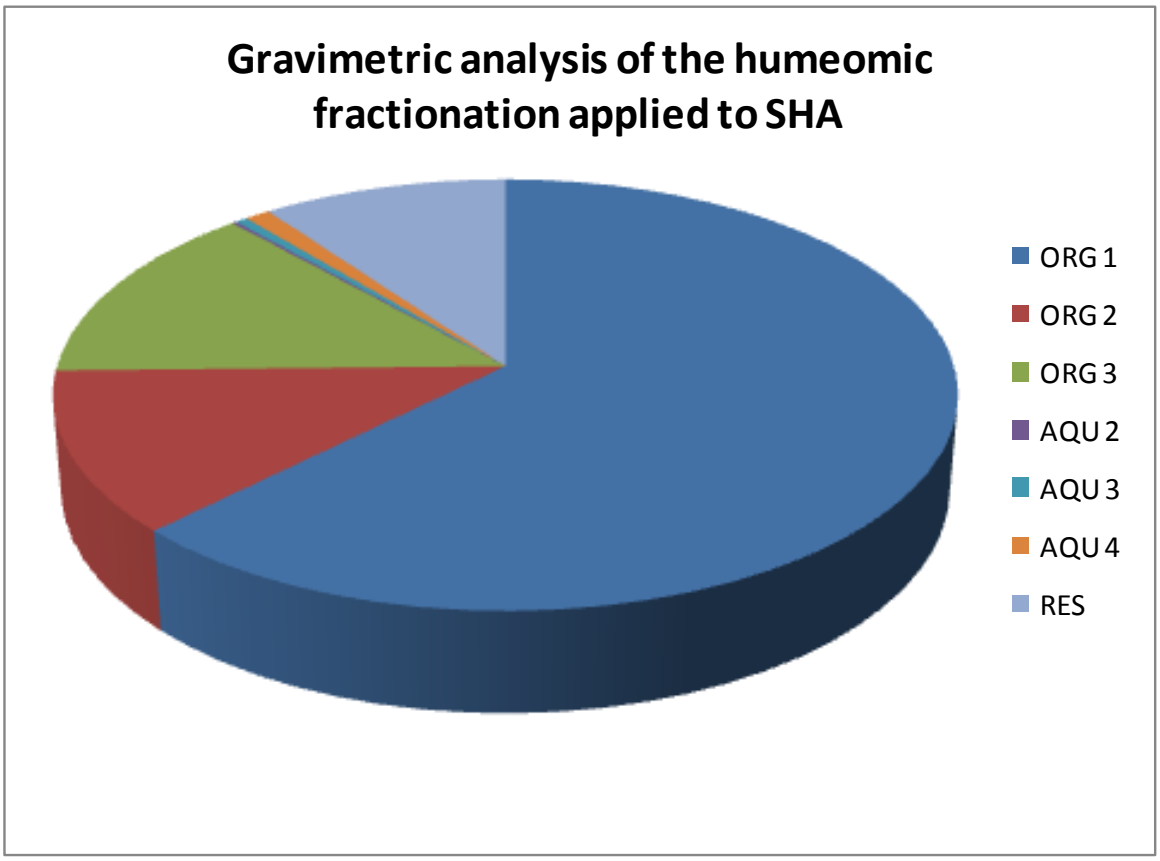



Figure III, 9: Percentage of gravimetric amounts for each of the samples extracted by sequential fractionation applying humeomics to SHA.

For the purpose of investigating the nature of the molecules present in the different fractions, some structural characterization was carried out. Taking into account that the AQU fractions were very low in quantity compared to the ORG and last RES fractions, the first three ORG fractions and the last RES fraction were partially characterized using GC-MS analysis, infrared spectroscopy (IR) and solid-state CPMAS-$^{13}$C NMR.

**General characterization of the main obtained fractions**

**GC-MS characterization**

Regarding GC-MS spectra as a hole, sililation residues and pyrydin (a derivatization reaction reagent) were detected before 10 min retention time (RT). From 15 min RT until 30 min RT, fatty acids and the internal standard (nonadeoic acid) were identified. After 30 min RT aromatic compounds were eluted out. A qualitative analysis was only carried out because calibration curves for each of the eluted substances were not prepared.

The main products identified in GC-MS analysis for ORG 1 sample are listed in Table III, 3. Long chain fatty acids were mostly detected in ORG 1 sample. At 22.65 min cinamic acid proposed structure was also detected.

Table III, 3. GC-MS spectra main peak associated structures for ORG 1 sample.

| Peak | RT(min) | Proposed structure |
|---|---|---|
| 1 | 29.26 | Octadecanoic acid |
| 2 | 25.24 | Hexadecanoic acid |
| 3 | 22.65 | Cinamic acid |

**ORG 1**

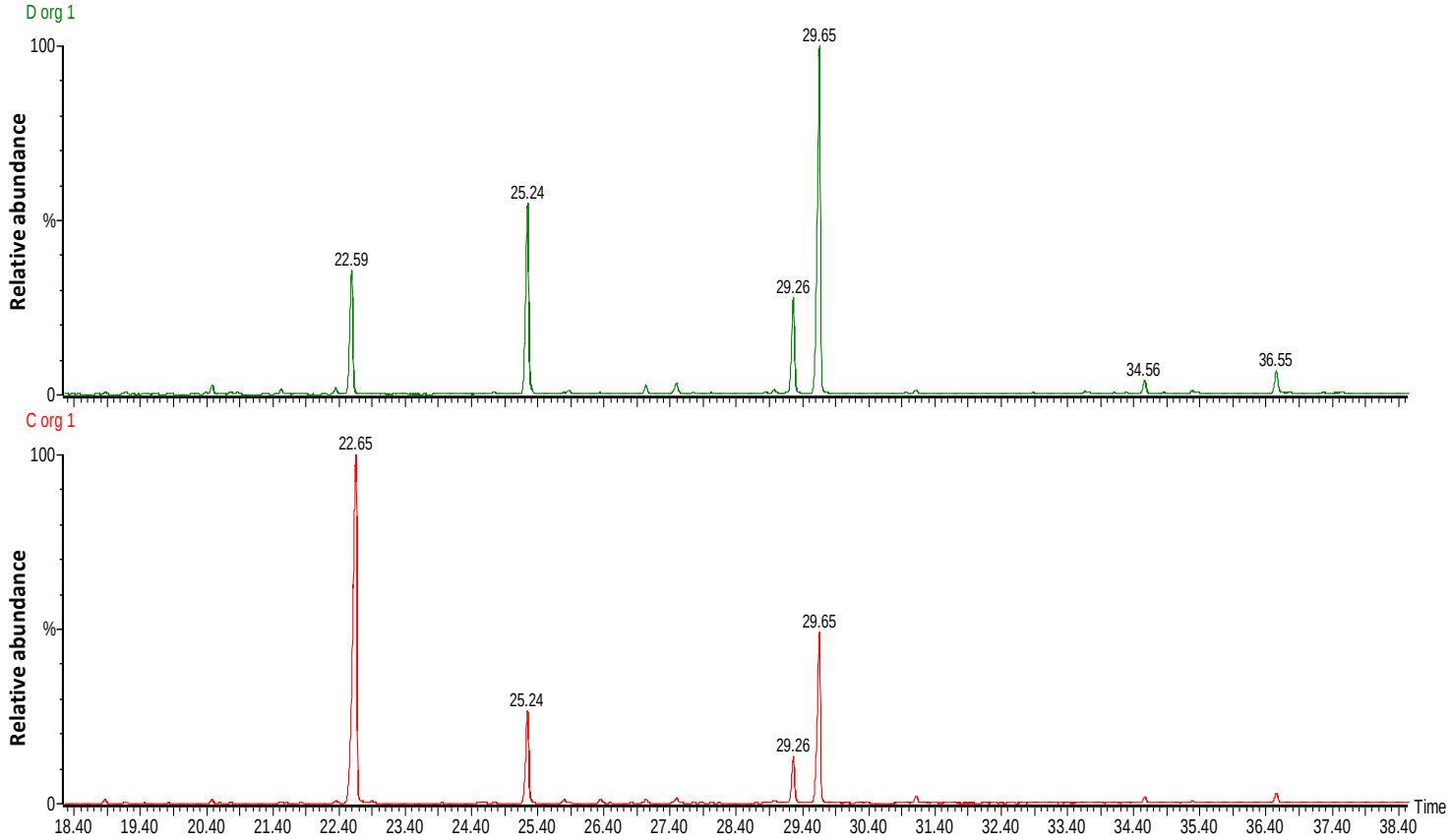



Figure III, 10. GC-MS spectra for ORG 1 sample. Two replicates are represented. At 29.65 min internal standard was detected (nonadecanoic acid).

For the ORG 2 fraction, some more products were identified compared to ORG 1. Mainly long chain fatty acids, identifying for the first time in this fraction the palmitoleic acid at 40.12 min RT, heptadecanoic acid at 27.03 min RT and pentadecanoic acid at 22.34 min RT (see table III, 4). Cinamic acid (figure III, 11), an unsaturated carboxylic acid, was identified again as in ORG 1 fraction. There were four compounds that NIST-MS library mass spectra software was not able to associate with any known molecular structure (table III, 4).

Table III, 4. GC-MS spectra main peak associated structures for ORG 2 sample.

| Peak | RT(min) | Proposed structure |
|---|---|---|
| 1 | 40.12 | No associated structure |
| 2 | 36.54 | No associated structure |
| 3 | 34.56 | No associated structure |
| 4 | 29.25 | Octadecanoic acid |
| 5 | 27.03 | Heptadecanoic acid |
| 6 | 25.23 | Hexadecanoic acid |
| 7 | 22.56 | Cinamic acid |
| 8 | 22.34 | Pentadecanoic acid |
| 9 | 19.46 | No associated structure |
| 10 | 16.43 | No associated structure |

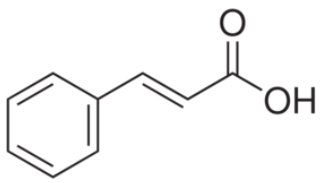



Figure III, 11: Cinamic acid structure.

**ORG 2**

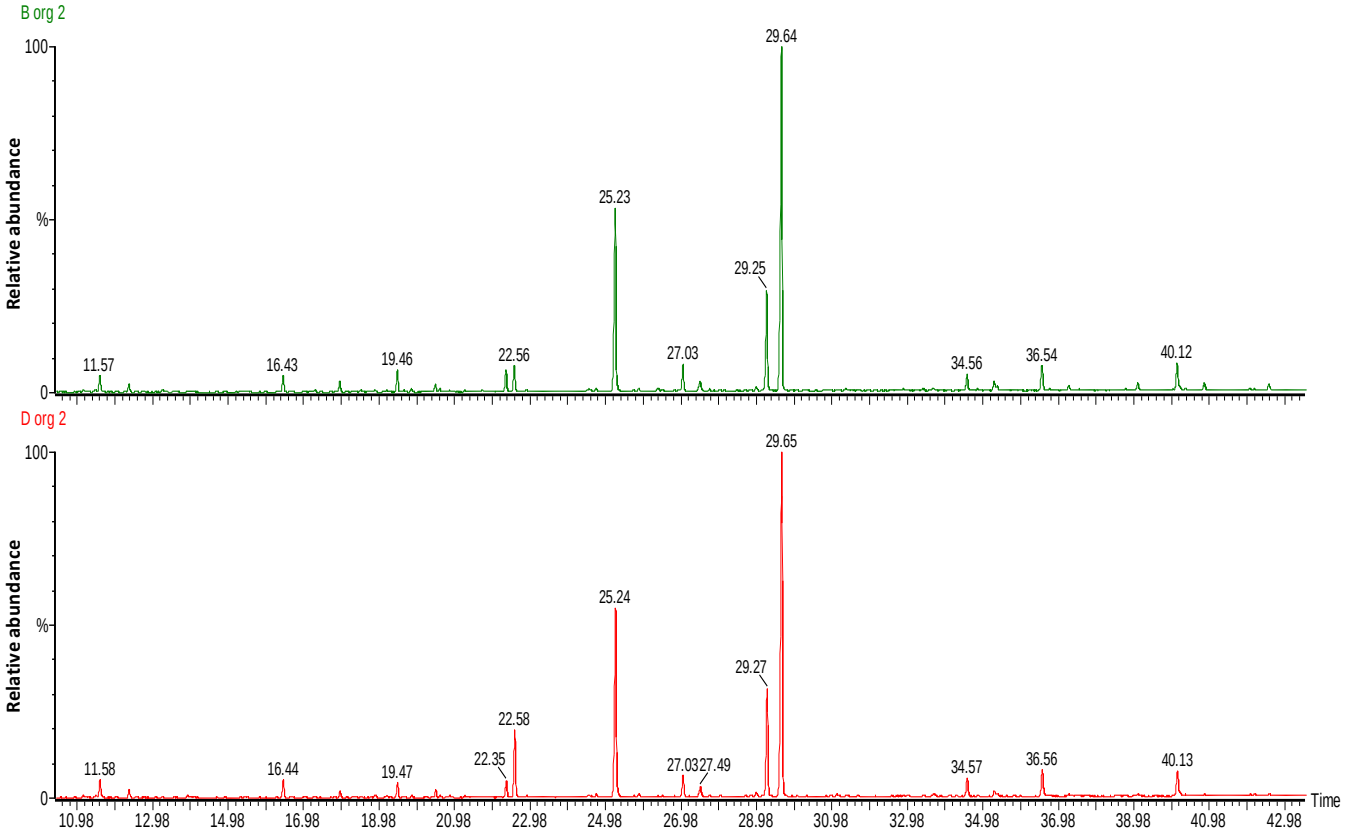



Figure III, 12. GC-MS spectra for ORG 2 sample. Two replicates are represented. At 29.65 min internal standard was detected (nonadecanoic acid)

Finally, for the ORG 3 GC-MS spectra molecular compound identification, there are slight differences compared to the previous ORG 2 analyzed spectra. Thus, ORG 3 cynamic acid disappeared from the spectra emerging a new aromatic compound that was identified at 31.11 min RT higher molecular weight (see table III, 5).

Table III, 5. GC-MS spectra main peak associated structures for ORG 3 sample.

| **Peak** | **RT(min)** | **Proposed structure** |
|---|---|---|
| 1 | 40.12 | No associated structure |
| 2 | 36.54 | No associated structure |
| 3 | 31.11 | Aromatic |
| 4 | 29.26 | Octadecanoic acid |
| 5 | 27.03 | Heptadecanoic acid |
| 6 | 25.23 | Hexadecanoic acid |
| 7 | 22.34 | Pentadecanoic acid |
| 8 | 16.43 | No associated structure |

**ORG 3**

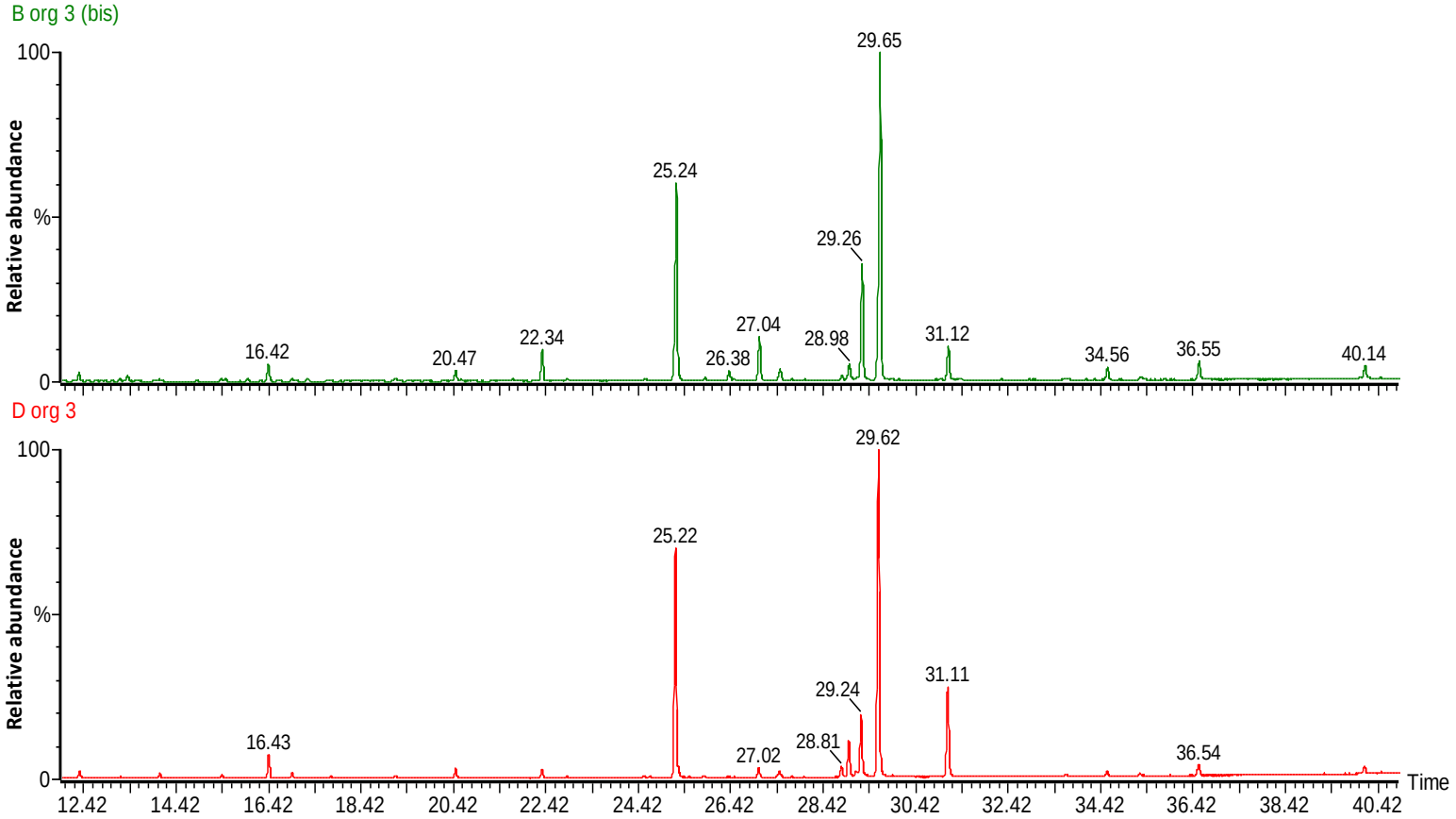



Figure III, 13. GC-MS spectra for ORG 3 sample. Two replicates are represented. At 29.25 min internal standard was detected (nonadecanoic acid).

## $^{13}$C-NMR spectrometry

Apart from the above presented GC-MS characterization data for ORG fractions, $^{13}$C-NMR analysis was carried out for the final residue obtained after humeomic sequential fractionation (RES 4). The main interest of this characterization is to investigate the potential changes in the aromatic and aliphatic features between the bulk SHA and RES 4. A tentative aromatic – aliphatic distribution for ORG plus AQU fractions was calculated by substraction of $^{13}$C-NMR spectra for RES 4 from bulk SHA.

**RES 4**

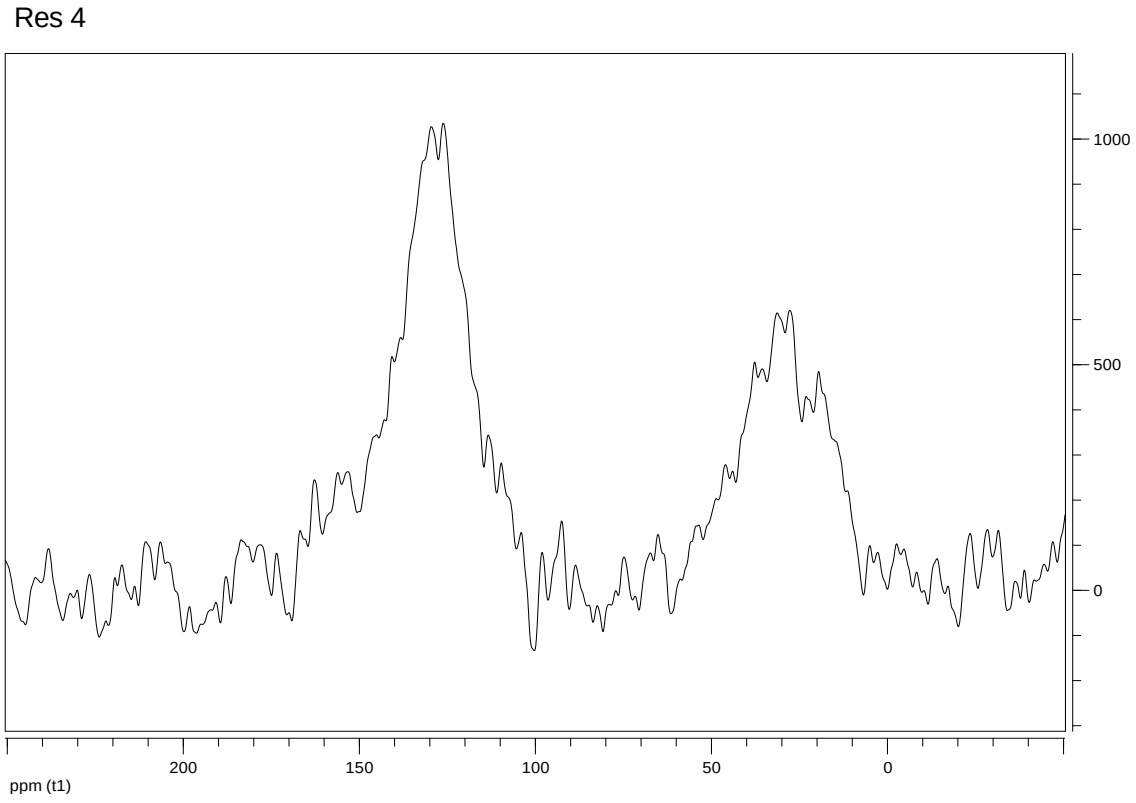



Figure III, 14: CPMAS SPECTRA $^{13}$C-NMR of final residue (RES 4) obtained after sequential humeomic fractionation.

Table III, 6: Quantification of area for each of the aliphatic or aromatic spectra region for initial SHA fraction (figure III, 2) or RES 4 fraction (figure III, 14). ORG and AQU fractions area is calculated by difference.

| | **Aliphatics** | **Aromatics** | **Aliphatics/Aromatics ratio** |
|---|---|---|---|
| Initial HA (Bulk) | 517209 | 610699 | 0.847 |
| ORG and AQU fractions | 291373 | 270703 | 1.076 |
| RES 4 (final residue) | 225836 | 339996 | 0.664 |

The quantification of the area for each of the aliphatic or aromatic region in the spectra show that the amount of aromatics was higher for the final residue (RES 4) and in the case of aliphatic compounds, the ORG and AQU fractions conserved larger amounts of these types of molecules on their structure. Results also indicated that the calculated aliphatic/aromatic ratio for ORG and AQU was higher than those for SHA and RES 4 (table III, 6). Data calculated by difference for the ORG and AQU fractions comprises in fact all the sequentially obtained fractions. Bearing in mind the proportion in weight of each fraction, it becomes clear that the aliphatic character showed by the hole fraction is mainly represented in the ORG fractions, (principally in ORG 1 fraction which represents 62.5% of the material).

**Biological activities of the fractions: relationship between chemical structure-biological activity of the main fractions in weight (ORG 1 and RES 1) after application of humeomics**

As explained before, the ability of the two fractions resulting from the first step of humeomics (ORG1 and RES1) to promote shoot and root growths was compared with that of bulk SHA. We selected these two fractions because they resulted from the breaking weak attraction forces like hydrogen bonds, van der Walls forces and hydrophobic effect (Nebbioso and Piccolo, 2011). Thus, both SRGR and RRGR as well as $Lp_r$ were measured after 24 and 72 hours from the onset of the treatments as described in chapter I and II.

**Chemical characterization of the main fractions ORG 1 and RES 1**

**FT- IR spectrometry**

FT-IR spectra were acquired for ORG 1 and remaining residue RES 1. In general, ORG 1 fraction showed lower intensity for O-H deformation of aliphatic O-H (1170 $cm^{-1}$) and C-O deformation of polysaccharides (1100 $cm^{-1}$). It also showed lower intensity on bands attributed to C=O stretching vibration 1685-1750 $cm^{-1}$ corresponding to COOH, ketones, aldehydes and esters and higher intensity between 2900 and 2950 $cm^{-1}$ caused by symmetric and asymmetric stretching vibrations of C-H in $CH_2$ and $CH_3$ groups (Prestch et al., 1998; Fuentes et al., 2007;) (Figure III, 15).

In relation to the residue sample (RES 1), it is remarkable the increase in 1000 $cm^{-1}$ region. It means higher intensity for O-H deformation of aliphatic O-H (1170 $cm^{-1}$) and C-O deformation of polysaccharides (1100 $cm^{-1}$). This spectra also exhibits higher intensity at COOH, ketone, aldehydes and esters stretching vibration 1685-1750 $cm^{-1}$ $cm^{-1}$ region and lower intensity for C-H stretching vibration for $CH_2$ or $CH_3$ groups (2900-2950 $cm^{-1}$) (figure III, 16).

Chen et al., (2006) proposed and index related to the aliphaticity/aromaticity relative character of a humic samples. This index may be obtained from the ratio between 2900-2950 $cm^{-1}$ intensity bands of C-H stretching vibration for $CH_2$ or $CH_3$ groups and

1600-1620 $cm^{-1}$ C-C stretching vibration in polycyclic aromatic hydrocarbons (PAHs). Thus, for ORG 1 fraction this index approximately corresponds to 1.2, whereas for RES 1 fraction this index is of 0.8. This result shows that ORG 1 fraction has a more aliphatic nature compared to RES 1 fraction.

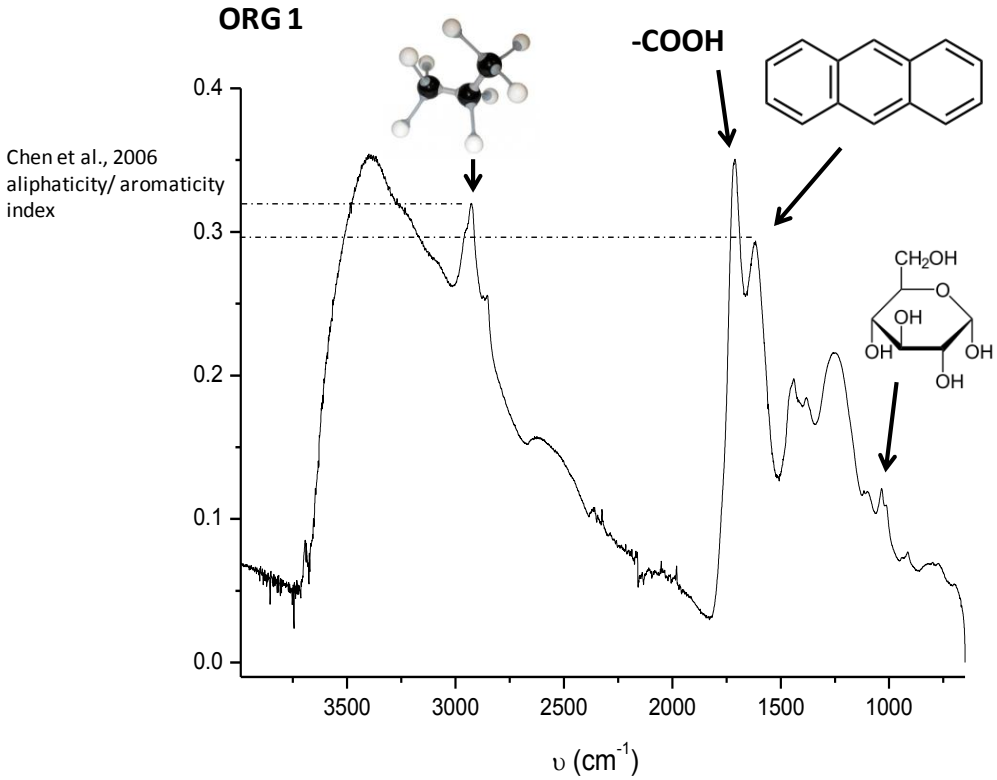



Figure III, 15. FT-IR spectra for ORG 1 fraction. The main considered peaks are marked with arrows. Two discontinuous lines mark the intensities to calculated aliphaticity/aromaticity index proposed by Chen et al. (2006).

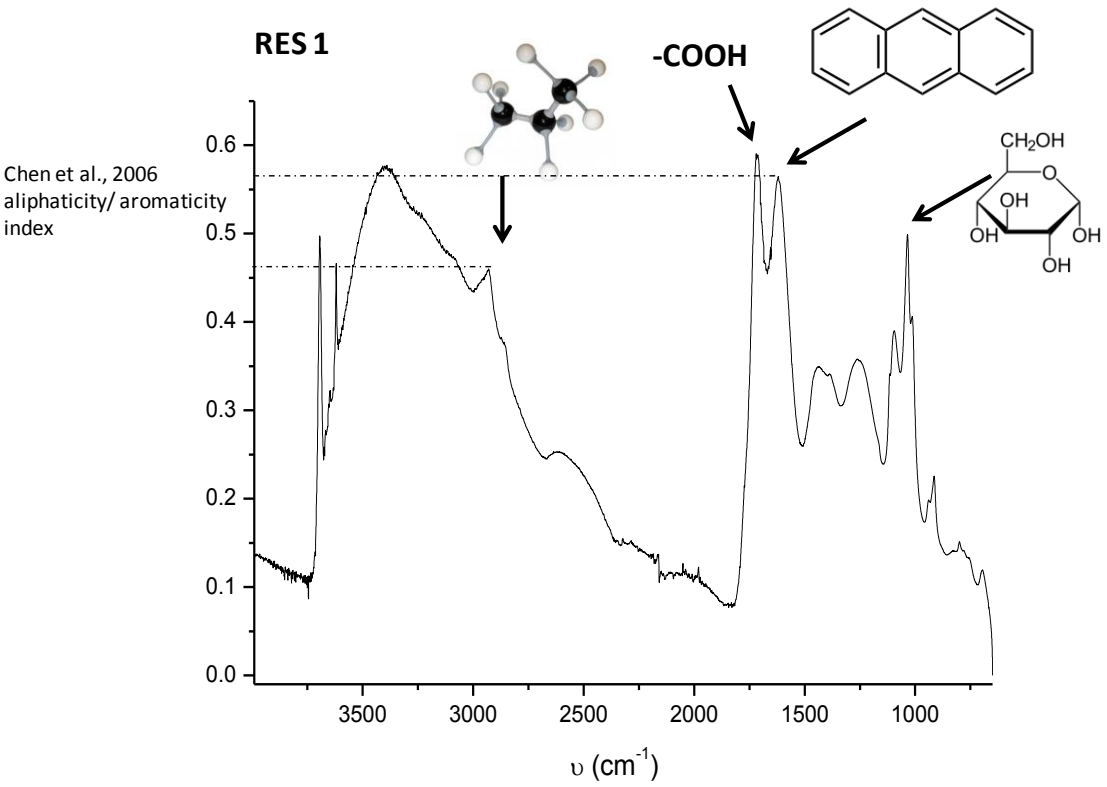



Figure III, 16. FT-IR spectra for RES 1 fraction. The main considered peaks are marked with arrows. Two discontinuous lines mark the intensities to calculated aliphaticity/aromaticity index proposed by Chen et al. (2006).

**Biological activity of the main fractions: ORG 1 and RES 1**

Regarding $Lp_r$, as it was previously reported by Olaetxea et al. (2015) (chapter II), SHA bulk fraction increased $Lp_r$ at both 24 and 72 hours compared to control plants. For the separated fractions instead, the activity was different. Both the residue fraction (RES 1) and ORG 1 fractions did not show increases on $Lp_r$ at 24h compared with control plants, but interestingly RES 1 showed higher $Lp_r$ than bulk SHA at 72 hours of treatment. However, ORG 1 fraction was not able to significantly increase $Lp_r$ compared with control plants (figure III, 17).

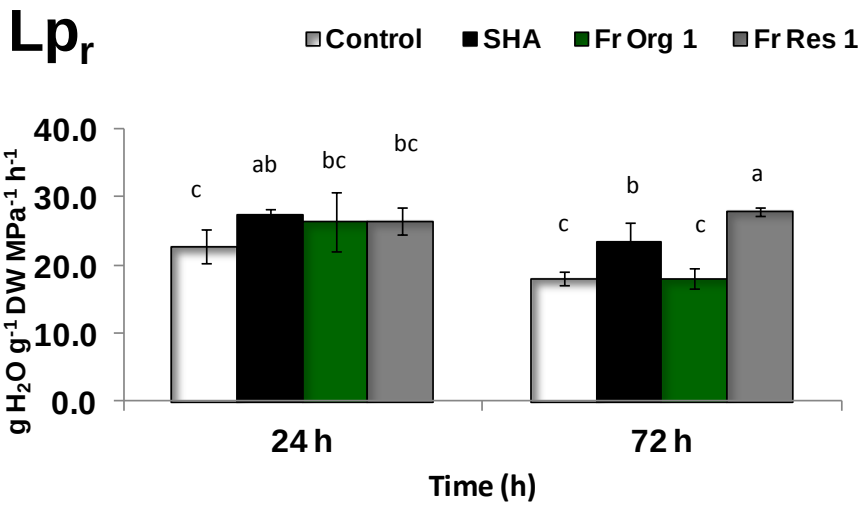



Figure III, 17. $Lp_r$ activity measured for control, SHA, ORG 1 and RES 1 treated plants after 24 and 72 hours from the onset of treatment.

In relation to the ability of both fractions to enhance root and shoot growths, data showed that the results are quite different depending on the fraction. In line with previous results, bulk SHA caused a significant increase in SRGR (table III, 7). However, whereas ORG 1 did not affect SRGR compared to the control or SHA treated plants, RES 1 fraction caused a very significant increase, even higher than that of bulk SHA (table III, 7).

Table III, 7. SRGR for control, SHA, ORG 1 and RES 1 treated plants between 24 and 72 hours of treatment.

| | **SRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|
| C | 115 c |
| SHA | 176 b |
| ORG 1 | 123 bc |
| RES 1 | 491 a |

With regard to root relative growth rate (RRGR), SHA treated plants showed significant increases compared to control plants. Again, as in the case of SRGR, RES 1 fraction showed significantly increased SRGR compared to control and bulk SHA treatment. In contrast to the effects on shoot growth, ORG 1 caused a significant increase in RRGR compared to control plants and similar to that for bulk SHA, but lower than that caused by RES1 (table III, 8).

Table III, 8. RRGR for control, SHA, ORG 1 and RES 1 treated plants between 24 and 72 hours of treatment.

| | **RRGR (mg $g^{-1}$ $d^{-1}$)** |
|---|---|
| C | 73 c |
| SHA | 128 b |
| ORG 1 | 121 b |
| RES 1 | 280 a |

**Discussion**

Considerable progress has been made over the last few years, providing information regarding the relationship between the structural nature of humic substances and the beneficial effects on plant growth promotion. However, due to the molecular complexity and diversity of HS, all this information remains rather fragmentary and has not been integrated into a general model. With the aim of studying HS composition, different fractionation processes and a wide range of chemical characterization techniques have been applied to HS. Among the different fractionation methods applied to HS, a chemical fractionation for a stepwise removal of molecules from complex humic matrices has been proposed and described by Nebbioso and Piccolo

(2011). This new fractionation method assumes the view of humic matrices as supramolecular structures whose complexity can be progressively reduced by breaking intra- and intermolecular interactions that define the bulk suprastructure. Indeed, connected with the fractionation processes, characterization techniques applied to HS such as, high-performance size exclusion chromatography (HPSEC) (Piccolo et al., 2002), pyrolysis-gas chromatography/mass spectrometry (GC/MS) (Martin et al., 1995; Almendros et al., 1997; Lehtonen et al., 2000), UV and fluorescence spectroscopy (Fuentes et al., 2006) or NMR spectroscopy (Simpson et al., 2001) among others, demonstrated the ability to partially elucidate the molecular composition of HS.

As for SHA, the operational humeomic-based fractionation method yielded a substantial amount of ORG 1 fraction, corresponding to 62.5% of the total bulk SHA. This fraction results from breaking weak intermolecular attraction forces in SHA whole supramolecule such as Van der Waals forces, hydrogen bonds or hydrophobic interactions. Based on a humeomics framework (Nebbioso and Piccolo 2011), this fact suggests that ORG 1 most likely involves a mixture of lipophilic molecules associated with a more hydrophilic core (RES 1). Thus ORG 1 might contain both an ensemble of individual molecules and supramolecular subunits. This fact was supported by the results obtained from GC-MS spectra, which was principally hydrophobic and composed of fatty acid (figure III, 10).

The nature of the rest of the ORG fractions (ORG 2 and ORG 3) was identified as being very similar to ORG 1, showing the existence of a range of fatty acids (figure III, 12 and 13). However, when referring to the conformation of the mentioned fractions, it is interesting to note that the reactions applied to extract ORG 2 and ORG 3 fractions were two trans-esterification reactions that, in theory, break chemical bonds with significant covalent character. Consequently, these fractions help characterize the structural features of a rather stable-molecular core. In any case, more work is needed to be carried out in order to complete the molecular characterization of these fractions by using more complementary derivatization processes.

Ignoring the analysis of the AQU fractions, because they are minor in quantity compared with the other fractions, it makes sense to mention that a last residue (RES

4), yielding close to 10% of the total amount of matter, was found in the bulk SHA (table III, 2). GC-MS spectra was not identified for the aforementioned fraction but based on $^{13}$C-NMR technique (figure III, 14), it can be concluded that it is the most aromatic fraction because aliphatic/aromatic ratios showed an evolution of increasing aromaticity between the final residue (RES 4) and the previously extracted fractions (ORG 1 until AQU 4), which corresponded to 0.664 (ORG1; AQU4) versus 1.076 (RES 4) (table III, 6). As for the molecular nature of the final residue (RES 4), it could probably correspond to a covalent bounded core because none of the previous applied extraction or cleavage reaction procedures was able to break it in smaller fractions.

With regard to physiological activity shown by the extracted fractions, it can be observed that only one of the two fractions (RES 1) was able to increase $Lp_r$ values after 72 hours from the onset of treatments (figure III, 17). Indeed, it showed significant increase not only compared with control plants but also compared with the bulk SHA treatment. In line with previous results (Olaetxea et al., 2015; chapter I), this increase in $Lp_r$ caused by RES1 was also associated with an increase in SRGR. However, ORG 1 fraction did not show any increase in either $Lp_r$ or SRGR (figure III, 17 and table III, 7). In line with the experiments presented in chapter one, where the mechanistic links between $Lp_r$ and SRGR was evidenced, it appears that the lipophilic fraction (ORG 1) surrounding a more hydrophilic core (RES 1) favors those interactions between the SHA and plant roots responsible for the enhancing action of SHA on shoot growth (table III, 7). With regard to the root growth promotion, RES 1 fraction, in line with the response found for shoot growth promotion, significantly increased root growth as well. For ORG 1 fraction, the response in roots was similar to SHA bulk treatment, showing a significant increase compared with control plants (table III, 8). These facts indicate that the mechanistic pathways behind the promoting effect of SHA on shoot or root developments relates to different structural features of SHA or are differently sensitive to them. In this regard, FTIR analyses of both ORG1 and RES1 demonstrated that RES 1 showed higher intensity for O-H deformation of aliphatic O-H (1170 $cm^{-1}$) and C-O deformation of polysaccharides (1100 $cm^{-1}$) than ORG1 did. RES 1 also showed higher intensity for COOH, ketone, aldehydes and esters stretching vibration bands (1685-1750 $cm^{-1}$) than ORG1 did. Chen et al (2006) proposed an index (figure III, 16)

that relates stretching vibration for $CH_2$ or $CH_3$ groups to C-C stretch modes of 1600-1620 $cm^{-1}$ in polycyclic aromatic hydrocarbons (PAHs). This index was lower for RES 1 fraction than for ORG 1 fraction, indicating the higher aromatic nature of RES 1 fraction. The ORG 1 fraction showed higher intensity for symmetric and asymmetric stretching vibrations of C-H in $CH_2$ and $CH_3$ groups (figure III, 15), thus showing more aliphatic character. These results suggest that the promotion shoot growth is likely more dependent on aromatic-related features, while root promotion is sensitive to both aliphatic and, more intensely, aromatic features of SHA.

However, further work is needed in order to demonstrate and better understand these relationships between SHA structural features and their ability to promote plant shoot and root developments.

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# CHAPTER IV

**SHA primary action in Arabidopsis roots involved significant changes in the regulation of major genes associated with root growth and plant development. Preliminary results.**

**Abstract**

*Arabidopsis thaliana* has been selected as a very useful candidate to study the genetic approach of different responses in plant systems. This choice is based on its small genome, only 5 chromosomes, its easiness to be transformed and generation of mutants. Also the small size and generation time has been important to be stablished as the primary model plant in modern science.

In the humics acid field, there are some reports that HS are able to alter gene expression in plants. To this aim a preliminary SHA root system genetic approach will be carried out in this chapter. In order to develop these experiments, the growth promoting effects of SHA on *Arabidopsis thaliana* will be evaluated. Two growing conditions will be used: the hydroponic conditions and the square plates growing conditions. The effects of SHA on plant growth were different depending on the method of cultivation system used. Arabidopsis seeds grown under hydroponic conditions showed significant differences in shoot and root growth for 0.5, 5 and 15 ppm of C from SHA. The concentration of 30 ppm of C of SHA did not show differences in shoot and root growth in hydroponics compared to control plants. However, for square plates, plants treated with a concentration of 100 ppm of C of SHA did not show increases in growth compared with controls.

SHA root application in square plates condition and further RNAseq determination showed that the main SHA up-regulated genes were related with Alpha-Expansin Gene Family (AT3G15370.1). This response together with AT5G57560.1 gene that encodes a cell wall-modifying enzyme, could be correlated with the previously studied (in chapter II) SHA root growth promotion ability. Indeed, AT1G14550.1 gene activation that encodes a peroxidase superfamily protein, could be possibly related with SHA exerted effects in peroxidase activity and ROS control (Calderín García et al., 2012). AT3G48360.1 encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway. Its biological function is related to the abscisic acid-activated signaling pathway, auxin-activated signaling pathway, response to abscisic acid and auxin among an extensive response network. The mentioned specific response could be correlated with SHA stimulated physiological response of

root-ABA synthesis linked to $Lp_r$ activation (chapter I) and root IAA-$H^+$-ATPase pathway (chapter II).

**Introduction**

Humic substances (HS) are extensively recognized as plant metabolism stimulators and plant growth promoters (Canellas et al., 2002; Chen et al., 2004; Dobbs et al., 2010; Mora et al., 2010, 2014a, 2014b). HS plant stimulation ability have been proved in several plant species such as maize (Trevisan et al., 2010), cucumber (Mora et al., 2010, 2014a, 2014b), wheat (Malik and Azam, 1985), *Brassica napus* (Jannin et al., 2012) and more interestingly for the purpose of this chapter in *Arbidopsis thaliana* (Baldotto et al., 2011; Trevisan et al., 2011). In this line, Arabidopsis plant species represents an appropriated tool to study the genetic response of the biological activity of humic susbtances due to the only five chromosomes composed genome and the short plant cycle. In order to identify the candidate genes involved in the regulation of the response to HS, some authors reported very interesting data. Trevisan et al. (2011), based on the detection of cDNA-AFLP markers, identified potential root genes involved in the biological activity of HS in Arabidopsis, concluding that HS exert their function through a complex transcriptional network partially connected to their well demonstrated auxin activity, but involving also IAA-independent signaling pathways. On the other hand, Jannin et al. (2012) reported that peat humic acid (HA) induced gene targets on *Brassica napus.* Furthermore, they characterize these expression changes and relate them to physiological events. Interestingly, four methabolic pathways were specifically affected in shoot and roots by HA treatment: general cell metabolism (10.6 % of the total differentially expressed genes), nitrogen and sulfur metabolism (corresponding to 6.6 % of the total differentially expressed genes), carbon metabolism and photosynthesis (6.1 % of the total) and stress responses related genes (6.1 %).

Taking all these data together, the aim of this chapter is, first of all, to evaluate the growth promoting effects of SHA on *Arabidopsis thaliana* by using two kind of growing methodologies, hydroponics (experiments carried out in collaboration with Verónica Mora) and square plates (experiments carried out in collaboration with Juan Carlos del

Pozo, Concepción Manzano, Sara Navarro and the team of CBGP). Next, in order to deeper study the nature of the effects corresponding to the primary action of SHA on root functionality and plant growth, we have investigated the effects of SHA at transcriptional level in Arabidopsis root using RNA seq approach (experiments carried out in collaboration with Carlos del Pozo ' s group and Berbara and García `s group).

## Material and methods

### Plant material and culture conditions for growth experiments in hydroponics

*Arabidopsis thaliana* was used for the first time in these experiments. In order to study the SHA dose that is appropriate for plant benefit, dose-response experiments were carried out. Thus, these SHA doses were proved in hydroponic conditions: 0.5, 5, 15 and 30 ppm of organich C from SHA.

*Arabidopsis thaliana* (ecotype Columbia) seeds were first of all sterilized by immersing them in 75% (v/v) NaOCl for 5 minutes. The sterilization procedure finishes by rinsing the seed on sterile deionized water. Seeds were kept for 48 hours at 4°C in the dark for stratification. Plants were first of all germinated in water and then grown (in Gibeaut medium) in a growth chamber. The medium was composed of: $Ca(NO_3)_2$ . $4H_2O$ (1.50mM), $KNO_3$ (1.25mM), $MgSO_4.7H_2O$ (0.75mM), $KH_2PO_4$ (0.50mM), $Na_2O_3Si.9H_2O$ (0.10mM), Fe- EDDHA (0.04mM), KCl (0.05mM), $MnSO_4.H_2O$ (0.01mM), $CuSO_4.5H_2O$ (0.0015mM), $ZnSO_4.7H_2O$ (0.002mM), $H_3BO_3$ (0.05mM), $(NH_4)_6Mo_7O_{24}$ (7.5 $10^{-4}$mM), $Na_2MoO_4 \cdot 2H_2O$ (0.0014mM).

Arabidopsis seeds were germinated in distilled water using rock wood. When seeds developed cotyledons, they were transferred to hydroponic solution. Plants were grown in receptacles containing 600 mL of solution for two plants. 10/14 light-dark photoperiod at 21 °C/18 ° were the conditions for the growth chamber. Different SHA concentrations were proved, 0.5, 5, 15 and 30 ppm of organic carbon from purified SHA specifically. Plants were treated after 8 days of growth in hydroponic solution. No precipitation was observed during the whole experiment. After 16 days of treatment, phenotypic changes were evaluated by fresh weight measurement of both root and shoot and some photos were also taken fort this purpose.

**Plant material and culture conditions for growth experiments in square plates.**

*Arabidpsis thaliana* (ecotype Columbia) seeds were first of all sterilized by immersing them in 75% (v/v) NaOCl for 5 minutes. The sterilization procedure finishes by rinsing the seed on sterile deionized water. Seeds were kept for 48 hours at 4°C in the dark for stratification. The growth medium was prepared adding half strength Murashige and Skoog with vitamins (2.2 g/L), MES (0.25g/L), sucrose (10g/L) and plant agar (10g/L) from (Duchefa). Growing solution was sterilized for ½ hours at 120°C. Approximately 100 mL of growing solution was added in each 12-cm square plate and they were vertically oriented for growth under 19/8 light-dark photoperiod at 21 °C/18 °C. The whole square plate was exposed to light. Seed were cultivated for 8 days and then transferred to new square plates that contained the treatment. The 100 ppm SHA treated square plates were prepared by adding a mixture of 3 mL of MES (0.5M), 15 mL of water and 1.5 mL of a 10000 ppm SHA solution (all solutions previously sterilized. This mix was added over a solidified medium in the squared plate and let on the laminar flow cabinet until the liquid was evaporated and a HS layer was formed over the growing medium. This SHA concentration was selected after studying different dose-response experiments (data not shown). The selected concentration was the one that best responded to plant benefit. For the control square plates, the same procedure was followed but without adding SHA solution. Plants were harvested after 3 days of treatment and RNA extraction procedure was applied to further RNAseq analyses. The two biological samples were prepared for these analyses and after the RNA extraction, RNA quality and quantity was analyzer with a Bioanalyzer (CNB genomic facilities, Madrid) (figure IV, 8 and 9).

For the evaluation of phenotypic changes and plant development, 3 repetitions of square plates were prepared for each of the treatment. In each square plate 24 seeds were grown directly treated from the germination. Plants were grown for 10 days. For the control square plates, the same procedure was followed but without adding SHA solution. Before harvesting the seedlings, root length measurements were carried out. Plants were weighted in groups of six plants (n=5) for root and shoot measurements.

**RNA sequencing technique**

Due to the interest of studying the molecular or genetic view of organisms in general, some technologies have been developed to characterize the transcriptome of the cells. The transcriptome is the unity of all the RNA molecules. Different technologies to analyze RNA expression, such as such as hybridation-based microarrays or Sanger sequencing-based methods (Yamada et al., 2003; Bertone et al., 2004; David et al., 2006; Nagalakshmi et al., 2010) can be used. RNA sequencing technology involves direct sequencing of complementary cDNA by using random oligo(dT) primers and it provides a more comprehensive understanding of the complexity of eukaryotic transcriptomes (Nagalakshmi et al., 2010) since it allows to analyze the expression level but also the identification of alternative splicing or the generation of new isoforms. For RNA seq determination two pairs of biological repetitions of control and 2 pairs for SHA were prepared and contrasted for data analysis.

**RNA extraction procedure**

RNA extraction procedure was carried out as indicated in RNeasy Mini Handbook. First of all, the RNA sample was adjusted to a volume of 100 µL with RNase-free water. 350 µL of RLT buffer were added and mixed well. Afterwards, 250 µL of ethanol (96-100 %) were added to the diluted RNA and it was mixed well by pipetting. The sample was then transferred to an RNeasy Mini spin column and placed in a 2 mL collection tube. It was centrifuged for 15 s at 10000 rpm. Flow-through was discarded. 500 µL of RPE buffer were added to RNeasy spin column and it was centrifuged again for 15 s at 10000rpm (this step was repeated twice). RNeasy spin column was placed in a new 1.5 mL collection tube. 30-50 µL of RNase-free water was directly added to the spin column membrane and it was centrifuged for 1 min at 10000 rpm. RNA purity and concentration was quantified byanalyses carried out with the Bioanalizer at the Genomics facility at the CNB (Madrid).

**Statistical analysis:**

Significant differences ($p < 0.05$) among treatments were calculated by using one-way analysis of variance (ANOVA) and the LSD Fisher *post hoc* test. All statistical test were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa USA).

## Results

### Evaluation of SHA treated plant development under hydroponic growing conditions

Regarding the SHA dose-dependent results for shoot and root growth, there were significant increase differences for both shoot and root compared to control plant for 0.5, 5 and 15 ppm of organic C from SHA (figure IV, 1 and figure IV, 2). When a concentration of 30 ppm of organic C was applied, there were no significant differences with control plants, neither in shoot nor in root (figure IV, 1 and figure IV, 2).

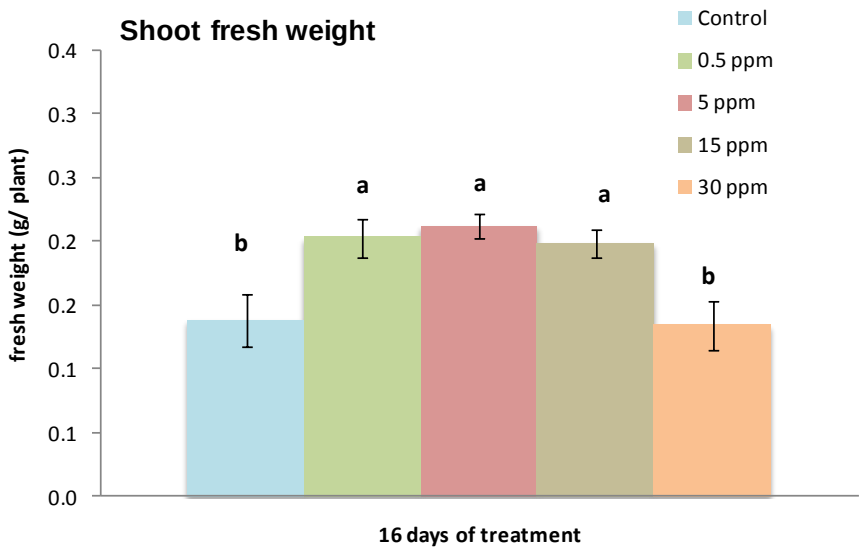


Figure IV, 1: Shoot fresh weight of Arabidopsis plants for different the SHA dose treatments after 16 days from the onset of treatment. Each weight value corresponds to the mean weight of a plant (n=10).

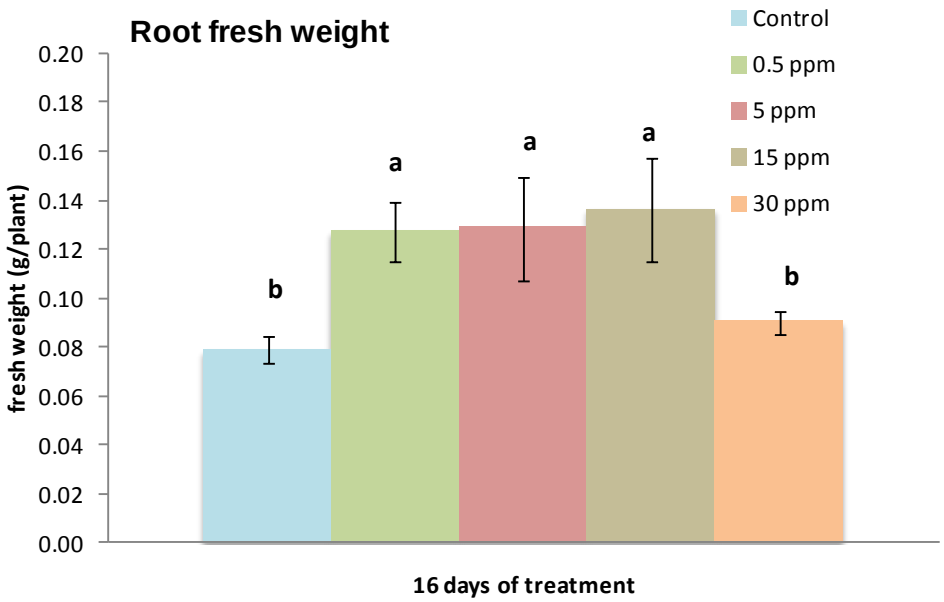


Figure IV, 2: Root fresh weight of Arabidopsis plants for the different SHA dose treatments after 16 days from the onset of treatment. Each weight value corresponds to the mean weight of a plant (n=10).

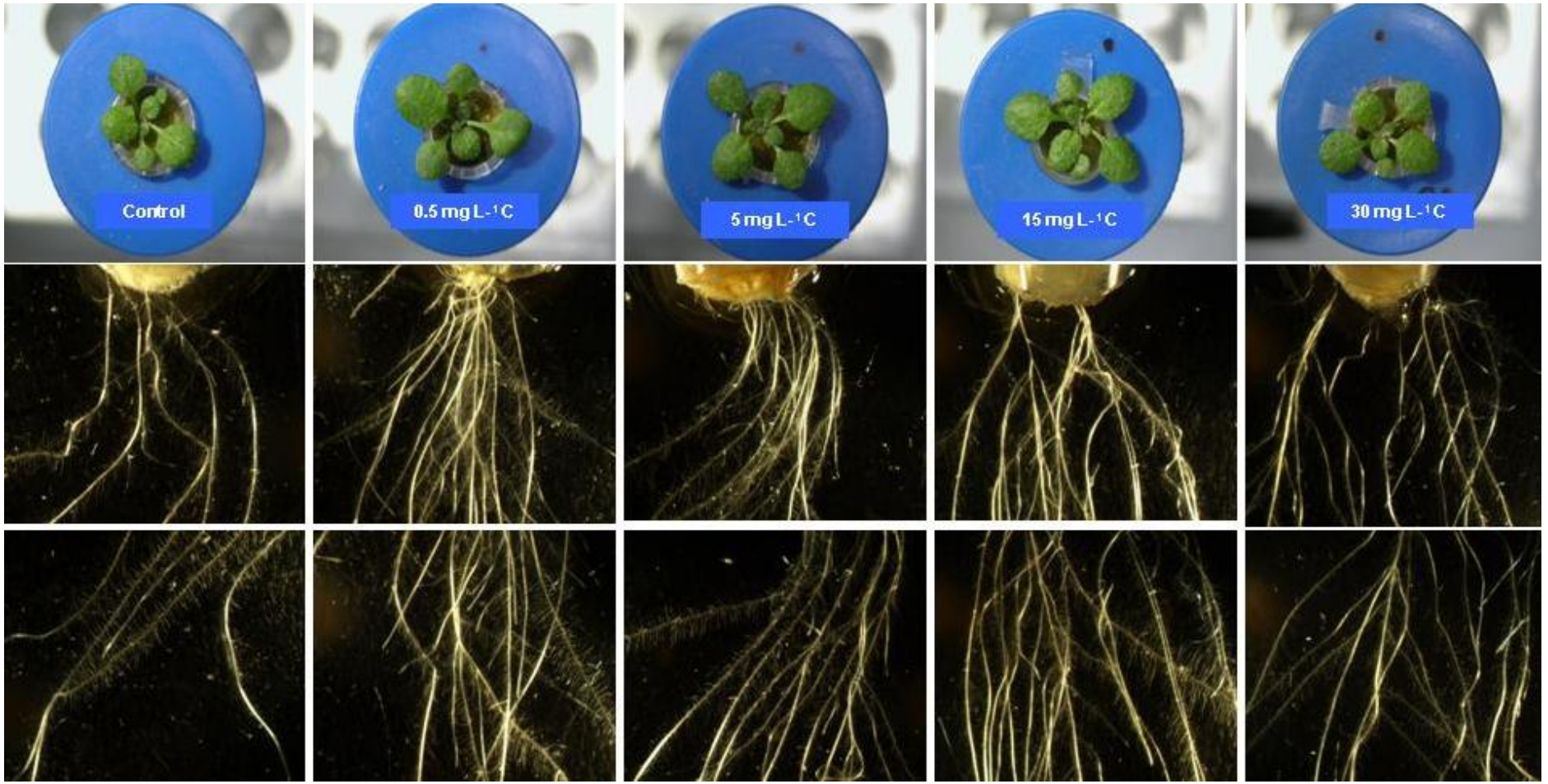


Figure IV, 3: These photos represent the development of *Arabidopsis thaliana* plants after 16 days of different SHA dose treatments in hydroponics. The first line represents the shoot of the plants. The second line of photos represents the root area of maturation and the last line the root area of elongation. Shoot photos were taken with a digital camera and the zoom of the root area using an optic microscope.

In relation to the phenotype evaluation of these plants (figure IV, 3), shoots of plants treated with 0.5, 5 or 15 ppm of SHA organic C seemed to be bigger than control plants. Apparently, 30 ppm treated plant presented less leaves than the other treated plants. Observed shoot data in photographs were well correlated with the fresh weight measurements (figure IV, 1), showing that 0.5, 5 or 15 ppm SHA treatments promoted plant shoot growth. However, for 30 ppm treated plants, there was not plant shoot promotion, neither on fresh weight results nor in photographs. Analyzing root photographs, in the maturation area (figure IV, 3, second line of photos), 0.5, 5 or 15 ppm SHA treatments, apparently, both the root mass and the secondary or lateral roots were increased. Also for the elongation area there were also differences in root mass and lateral root emergency for 0.5, 5 or 15 SHA treatments compared with control plants or SHA 30 ppm treated plants. These results suggest that this SHA concentration (30 ppm) might be toxic for Arabidopsis plants grown under this growing condition, that is why root area could have less root-shoot mass and less lateral root emergency.

**Evaluation of SHA treated plant development under square plates growing conditions**

For this experiment, Arabidopsis seeds were grown in square plates. These seeds were directly germinated under the treatment (in the case of SHA treated square plates) as explained in material and methods. In relation to the shoot and root fresh weight measurements, there were no significant differences between control and SHA treatments neither in roots nor in shoots (figure IV, 4)

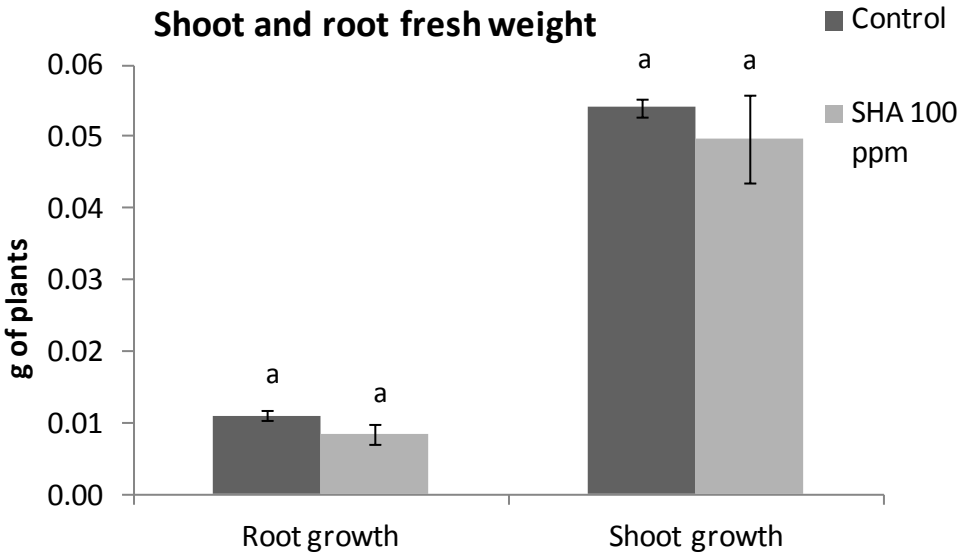


Figure IV, 4: Shoot and root fresh weight determination after 10 days of treatment. Each of the mean weight value corresponds to the sum of six plants (n= 5).

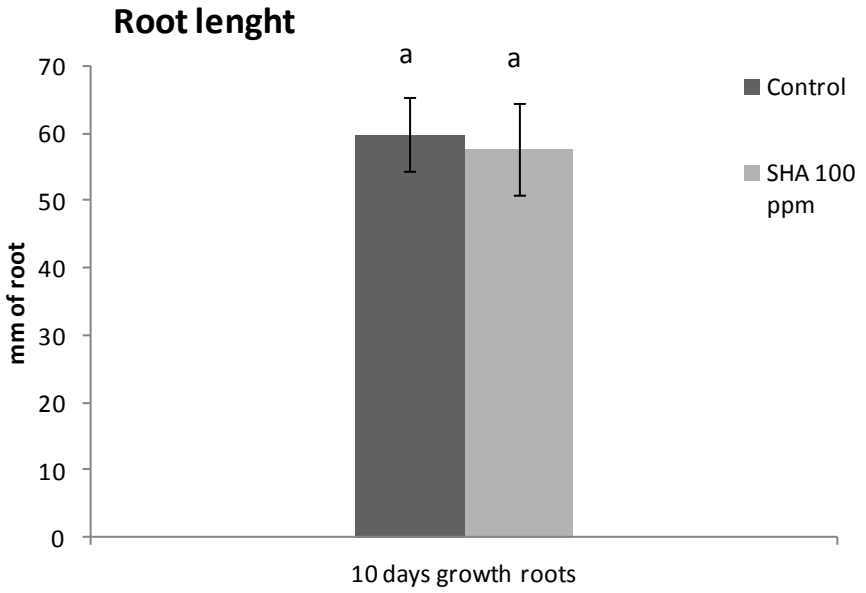


Figure IV, 5: Root length measurements after 10 days of growth. SHA treated seeds were directly germinated in SHA.

In order to include another approach to study more in depth the influence in growth of SHA treatment under square plates growing conditions, root length measurements were carried out. As it can be observed in figure IV, 5, there were no significant differences between control and SHA treated roots.

Figure IV, 6: Arabidopsis thaliana control plants grown in square plates. 8 days of growth. This square plate corresponds to the RNAseq experiment.

Figure IV, 7: *Arabidopsis thaliana* SHA treated plants grown in square plates. 8 days of growth (3 days of treatment). This square plate corresponds to the RNAseq experiment.

**RNAseq analysis**

Among all the positively expressed genes, a deeper searching on the biological process or the molecular function in which they are involved was carried out for the genes that were more than two fold expressed after SHA plant treatment (table IV, 1). RNAseq analysis also showed the SHA exerted negatively expressed genes (table IV, 2).

The information published in arabidopsis.org website was used to search for the biological or molecular features for each more than two fold up or down regulated genes.

AT4G38420.1 (12.28 fold change) gene regulation is related with oxidoreductase activity and copper ion binding. It is involved in oxidation and reduction processes. However, the identification of AT4G38420.1 gene at these samples located the function of the mentioned gene in plant leaves, cotyledons, guard cells (and some

more locations in leaves). All the samples that were analyzed by RNAseq were root samples, so it could be that the identification of this gene contains some errors.

AT3G15370.1 (7.28 fold change) gene belongs to an Alpha-Expansin Gene Family. These genes are related to the plant cell wall loosening, plant cell wall organization and with the undimensional cell growth.

AT1G14550.1 (3.06 fold change) gene encodes a peroxidase superfamily protein. This gene is related to oxidation and reduction processes and with responses to oxidative stress.

AT2G41100.1 (2.55 fold change) gene encodes a calmodulin-like protein, with six potential calcium binding domains. The expression is induced by touch and darkness. The expression is located in growing regions of roots, in root/to shoot junctions etc.

AT2G14960.1(2.44 fold change) gene encodes a protein similar to IAA-amino synthases.

AT1G61750.1 (2.36 fold change) encodes a receptor-like protein kinase-related family protein.

AT5G57560.1 (2.18 fold change) gene encodes a cell wall-modifying enzyme, rapidly up regulated in response to environmental stimuli.

AT3G48360.1 (2.08 fold change) encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway. Its biological function is related to the abscisic acid-activated signaling pathway, to the auxin-activated signaling pathway, related to the regulation of transcription, DNA-templated, response to abscisic acid, response to auxin, response to carbohydrates, response to cold, response to hydrogen peroxide, response to jasmonic acid, response to nitrate, response to salicylic acid, response to salt stress, response to wounding, response to sugar mediated signaling pathway etc.

AT2G05540.1 (2.03 fold change) encodes a glycine-rich protein family. However, as for the identification of AT4G38420.1 gene, the function of this gene is located in the leaf

or leaf related locations. Maybe the identification process could have failed for this gene because root samples were checked in these experiments.

Among the negatively expressed genes, the following genes were considered because they were two fold under-regulated.

AT2G34430.1 (-11.51 fold change) gene encodes a light-harvesting chlorophyll-protein complex II subunit B1. The biological processes in which this encoded protein is involved are the photosynthesis, the light harvesting photosystem I and II and the response to light stimulus. The plant structure in which AT2G34430.1 gene is expressed is on cotyledons, in guard cells and in shoot system in general. RNAseq analysis were carried out in root samples, so it seems that there could be an error on the identification of this specific gene.

AT4G14690.1 (-3.14 fold change) gene encodes ELIP2_Chlorophyll A-B binding family protein involved in cellular response to light processes. In this case, although in general this gene is expressed in shoot system, it can be also expressed in root system.

AT2G22590.1 (-3.011 fold change) gene encodes a UDP-Glycosyltransferase superfamily protein involved in transferase activity, transferring glycolsyl groups metabolic processes.

AT1G76780.1 (-2.34 fold change) gene encodes a HSP20-like chaperones superfamily protein.

AT5G41790.1 (-2.32 fold change) gene encodes a CIP1, COP1-interactive protein 1 encodes a protein that physically interacts specifically with the putative coiled-coil region of COP1. The expression of this gene is sometimes found in roots but some other times not.

AT5G62210.1 (-2.21 fold change) gene encodes Embryo-specific protein 3, (ATS3), involved in any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a karrikin stimulus. Its expression is located in shoot system but the RNAseq determined sample was from the root system.

AT1G23160.1 (-2.19 fold change) gene encodes an auxin-responsive GH3 family protein involved in auxin response.

AT1G29000.1 (-2.18 fold change) gene encodes a heavy metal transport/detoxification superfamily protein involved in cellular transition metal ion homeostasis and in metal ion transport in general.

Table IV, 1: Partial list of positively expressed Arabidopsis thaliana root genes. Fold change indicated that gene is specifically over-expressed in response to SHA treatment.

| GeneID | | Fold Change |
|---|---|---|
| AT4G38420.1 | sks9__SKU5 similar 9 | 12.28995 |
| AT3G15370.1 | ATEXP12_ATEXPA12_ATHEXP ALPHA 1.24_EXP12_EXPA12__expansin 12 | 7.284331 |
| AT1G14550.1 | Peroxidase superfamily protein | 3.067558 |
| AT2G41100.1 | ATCAL4_TCH3__Calcium-binding EF hand family protein | 2.557439 |
| AT2G14960.1 | GH3.1__Auxin-responsive GH3 family protein | 2.44004 |
| AT1G61750.1 | Receptor-like protein kinase-related family protein | 2.361242 |
| AT5G57560.1 | TCH4_XTH22__Xyloglucan endotransglucosylase/hydrolase family protein | 2.188048 |
| AT3G48360.1 | ATBT2_BT2__BTB and TAZ domain protein 2 | 2.081844 |
| AT2G05540.1 | Glycine-rich protein family | 2.037391 |
| AT3G15450.1 | Aluminium induced protein with YGL and LRDR motifs | 1.855191 |
| AT4G04450.1 | AtWRKY42_WRKY42__WRKY family transcription factor | 1.832137 |
| AT1G33055.1 | unknown protein | 1.818581 |
| AT2G25900.1 | ATCTH_ATTZF1__Zinc finger C-x8-C-x5-C-x3-H type family protein | 1.775159 |
| AT5G20250.4 | DIN10_RS6__Raffinose synthase family protein | 1.770429 |
| AT5G23990.1 | ATFRO5_FRO5__ferric reduction oxidase 5 | 1.756733 |
| AT3G48390.1 | MA3 domain-containing protein | 1.752955 |
| AT5G20860.1 | Plant invertase/pectin methylesterase inhibitor superfamily | 1.749953 |
| AT2G31945.1 | unknown protein | 1.683995 |
| AT5G63160.1 | BT1__BTB and TAZ domain protein 1 | 1.666707 |
| AT4G08950.1 | EXO__Phosphate-responsive 1 family protein | 1.649294 |
| AT1G30730.1 | FAD-binding Berberine family protein | 1.649109 |
| AT1G76410.1 | ATL8__RING/U-box superfamily protein | 1.646121 |

Table IV, 2: Partial list of negatively expressed Arabidopsis thaliana root genes. Fold change indicated that gene is specifically under-expressed in response to SHA treatment.

| GeneID | | Fold Change (SHA vs C) |
|---|---|---|
| AT2G34430.1 | LHB1B1_LHCB1.4__light-harvesting chlorophyll-protein complex II subunit B1 | -11.515483 |
| AT1G56660.1 | unknown protein | -4.5597185 |
| AT4G14690.1 | ELIP2__Chlorophyll A-B binding family protein | -3.1498904 |
| AT2G22590.1 | UDP-Glycosyltransferase superfamily protein | -3.0116097 |
| AT5G40450.1 | unknown protein | -2.8239357 |
| AT5G52280.1 | Myosin heavy chain-related protein | -2.3842073 |
| AT1G76780.1 | HSP20-like chaperones superfamily protein | -2.3403639 |
| AT5G42710.1 | unknown protein; INVOLVED IN: biological_process unknown. | -2.3331312 |
| AT5G41790.1 | CIP1__COP1-interactive protein 1 | -2.325989 |
| AT2G22795.1 | unknown protein | -2.3000727 |
| AT5G62210.1 | Embryo-specific protein 3, (ATS3) | -2.2116583 |
| AT1G23160.1 | Auxin-responsive GH3 family protein | -2.1982731 |
| AT1G29000.1 | Heavy metal transport/detoxification superfamily protein | -2.1804034 |
| AT1G60640.1 | unknown protein | -2.1652338 |
| AT5G60030.1 | unknown protein | -2.1367103 |
| AT3G22840.1 | ELIP_ELIP1__Chlorophyll A-B binding family protein | -2.1284895 |
| AT5G24880.1 | BEST Arabidopsis thaliana protein match is: calmodulin-binding protein-related (TAIR:AT5G10660.1) | -2.1033913 |
| AT5G42600.1 | MRN1__marneral synthase | -2.0359344 |
| AT1G15940.1 | Tudor/PWWP/MBT superfamily protein | -2.0275651 |
| AT2G40250.1 | SGNH hydrolase-type esterase superfamily protein | -1.9976511 |
| AT5G55660.1 | DEK domain-containing chromatin associated protein | -1.9767901 |
| AT4G26630.2 | DEK domain-containing chromatin associated protein | -1.9402131 |
| AT4G15480.1 | UGT84A1__UDP-Glycosyltransferase superfamily protein | -1.9162105 |
| AT3G21560.1 | UGT84A2__UDP-Glycosyltransferase superfamily protein | -1.9089423 |
| AT3G02620.1 | Plant stearoyl-acyl-carrier-protein desaturase family protein | -1.8798699 |
| AT2G28720.1 | Histone superfamily protein | -1.8778089 |
| AT3G53350.1 | RIP4__ROP interactive partner 4 | -1.8729414 |
| AT4G00930.1 | CIP4.1__COP1-interacting protein 4.1 | -1.8407602 |
| AT4G39190.1 | unknown protein | -1.8376085 |
| AT5G60530.1 | late embryogenesis abundant protein-related / LEA protein-related | -1.8353696 |
| AT1G12150.1 | Plant protein of unknown function (DUF827) | -1.8291903 |
| AT5G08050.1 | Protein of unknown function (DUF1118) | -1.8271275 |
| AT3G19050.1 | POK2__phragmoplast orienting kinesin 2 | -1.8214263 |
| AT1G63300.1 | Myosin heavy chain-related protein | -1.8211554 |
| AT4G17670.1 | Protein of unknown function (DUF581) | -1.8169315 |
| AT4G36520.1 | Chaperone DnaJ-domain superfamily protein | -1.803968 |
| AT1G64330.1 | myosin heavy chain-related | -1.7971921 |
| AT1G65010.1 | Plant protein of unknown function (DUF827) | -1.7937224 |
| AT3G05900.1 | neurofilament protein-related | -1.7931828 |
| AT1G68790.1 | CRWN3_LINC3__little nuclei3 | -1.7843542 |
| AT5G41140.1 | Myosin heavy chain-related protein | -1.7797287 |
| AT3G55120.1 | A11_CFI_TT5__Chalcone-flavanone isomerase family protein | -1.7770212 |
| AT5G16730.1 | Plant protein of unknown function (DUF827) | -1.7442139 |
| AT1G13220.2 | CRWN2_LINC2__nuclear matrix constituent protein-related | -1.7410817 |
| AT1G34760.1 | GF14 OMICRON_GRF11_RHS5__general regulatory factor 11 | -1.7306895 |
| AT2G44200.1 | CBF1-interacting co-repressor CIR, N-terminal;Pre-mRNA splicing factor | -1.721052 |
| AT4G12470.1 | AZI1__azelaic acid induced 1 | -1.7210092 |
| AT1G65060.1 | 4CL3__4-coumarate:CoA ligase 3 | -1.7092189 |

Table IV, 2: continued

| GeneID | | Fold Change (SHA vs C) |
|---|---|---|
| AT4G00670.1 | Remorin family protein | -1.7073263 |
| AT1G05320.2 | FUNCTIONS IN: molecular_function unknown | -1.7071529 |
| AT5G40340.1 | Tudor/PWWP/MBT superfamily protein | -1.7070082 |
| AT3G56290.1 | unknown protein | -1.6955293 |
| AT3G02930.1 | Plant protein of unknown function (DUF827) | -1.6940768 |
| AT3G19370.3 | Plant protein of unknown function (DUF869) | -1.691055 |
| AT4G27595.1 | Plant protein of unknown function (DUF827) | -1.6903913 |
| AT5G22320.1 | Leucine-rich repeat (LRR) family protein | -1.6897717 |
| AT2G47460.1 | ATMYB12_MYB12_PFG1__myb domain protein 12 | -1.6889768 |
| AT3G46270.1 | receptor protein kinase-related | -1.6855435 |
| AT1G73860.1 | P-loop containing nucleoside triphosphate hydrolases superfamily protein | -1.6827824 |
| AT5G37550.1 | unknown protein | -1.6700306 |
| AT1G10320.1 | Zinc finger C-x8-C-x5-C-x3-H type family protein | -1.6674385 |
| AT5G04120.1 | Phosphoglycerate mutase family protein | -1.6660688 |
| AT2G32240.1 | FUNCTIONS IN: molecular_function unknown | -1.6619715 |
| AT4G31320.1 | SAUR-like auxin-responsive protein family | -1.6605432 |
| AT4G37820.1 | unknown protein | -1.6552876 |
| AT2G03720.1 | MRH6__Adenine nucleotide alpha hydrolases-like superfamily protein | -1.652034 |
| AT5G55820.1 | WYR__CONTAINS InterPro DOMAIN/s: Inner centromere protein, ARK-binding region (InterPro:IPR0 | -1.6503448 |
| AT4G38400.1 | ATEXLA2_ATEXPL2_ATHEXP BETA 2.2_EXLA2_EXPL2__expansin-like A2 | -1.6489055 |
| AT5G65900.1 | DEA(D/H)-box RNA helicase family protein | -1.6436627 |
| AT1G20970.1 | FUNCTIONS IN: molecular_function unknown | -1.6282675 |
| AT5G63550.2 | DEK domain-containing chromatin associated protein | -1.62783 |
| AT1G43160.1 | RAP2.6__related to AP2 6 | -1.6272823 |
| AT4G14760.1 | kinase interacting (KIP1-like) family protein | -1.6254299 |

**Discussion**

Although the SHA treatment was not applied for the same time duration (16 days of treatment in hydroponic conditions against 10 days of treatment in square plates) in both, hydroponic or square plates growing conditions, the results were different. Arabidopsis seedlings grown under hydroponic conditions showed significant differences in shoot and root growth for 0.5, 5 and 15 ppm of C from SHA (figures IV, 1 and 2). However, in square plates growing conditions, with a concentration of 100 ppm of C from SHA applied, there were not significant differences between control and SHA treated plants either for shoot or root weight nor for root length measurement (figures IV, 4 and 5). This fact suggests that the growing conditions affect SHA treatment efficiency. In this line, it is important to take into account that in hydroponic conditions plants generally grow faster because of the high availability of nutrients and water. In square plates, however, as the medium is composed by agar, it seems logical to think that the availability of nutrients will be shorter than in hydroponics. In relation to SHA treatments specifically, a possible explanation of why plants showed significant differences in growth under hydroponics against the results in square plates would be that in hydroponics the availability or reactivity of SHA molecules would be higher than in square plate conditions and consequently the physiological changes in growth would be observed later under square plate conditions. This is a hypothesis that needs to be studied in detail in further experiments.

As SHA altered gene regulation, some of the described activities could be related with part of the mechanism of action described in previous chapters or with the effects previously reported by other authors. AT3G15370.1 gene function, identified as member of an Alpha-Expansin Gene Family, is related to the plant cell wall loosening, plant cell wall organization and with the undimensional cell growth. A published study (Cosgrove et al., 2000) reported that during plant growth, cells secrete this protein (expansin), which unlocks the network of wall polysaccharides, permitting turgor-driven cell enlargement. Studying the acid-growth theory in detail, the activation of AT3G15370.1 gene function by SHA could be explained by a series of facts. First of all, as reported by Mora et al. (2014b) SHA induced IAA-root biosynthesis. Based on the acid-growth theory, the growth-hormone auxin (IAA), in an activated state, enhances

the activity of this $H^+$ -ATPase thus causing a lowering of the apoplastic pH (Hager, 2003). Together with this event, the reduced pH in the wall activates cell-wall-loosening enzymes and initiates the enlargement of the cell (Hager, 2003). This is the main event that could be correlated with the activation of AT3G15370.1 gene function and could explain root growth. Indeed, there is another up-regulated gene expression AT5G57560.1 gene that encodes a cell wall-modifying enzyme, which could be correlated with the above mentioned cell enlargement.

As another explanation of cell wall elongation, it was proposed that the primary driving force for the stretching of the cell wall was to increase turgor pressure as a result of increased uptake of osmotically active material (ions and sugars) together with water into the vacuole (Hager, 2003). This phenomenon could be related with the need of enhancing water and nutrients uptake by $Lp_r$ activity (that permits water uptake and it is enhanced by SHA application, chapter I).

AT1G61750.1 encodes a receptor-like protein kinase-related family protein. There are some recent studies that show the potential role of HA from vermicompost on eliciting signaling dependent on $Ca^{+2}$-dependent protein kinase (CDPK) at early stages of lateral plant root development (Ramos et al., 2015)

Nevertheless, more studies are needed in order to relate specifically the genetic expression involved in the mention cell elongation phenomena and the physiological responses of $Lp_r$ or $H^+$-ATPase activity. These studies could be developed using this specific gene mutants.

Among the SHA activated root genes, AT2G14960.1 was identified as another gene that encodes a protein similar to IAA-amino synthases. Some IAA–amino synthetases maintain auxin homeostasis by conjugating excess IAA to amino acids (Ding et al., 2008). Considering that SHA root action involves an root-IAA biosynthesis linked to $H^+$-ATPase activation (chapter II), it might be that the prompt repression of this gene conjugating IAA produces the accumulation on root IAA that is necessary for SHA action. In fact this resgulation of IAA biosynthesis homeostasis could be related with the regulation of cell wall elongation promoted by AT3G15370.1 gene function that codifies expansins, taking into account that these factors are activated by IAA.

In relation to AT1G14550.1 gene activation, that encodes a peroxidase superfamily protein, it was previously showed in rice plants grown in vermicompost, which contains HA induced peroxidase enzimatic activity, leading to a reduction in $H_2O_2$ content and ensuring a greater conservation of membrane permeability and preventing oxidative stress (Calderín García et al., 2012). The involvement of AT1G14550.1 gene in SHA response also corroborates the previously described SHA effects on peroxidase activity. Indeed, it is interesting to highlight that the origin of the applied HA in both studies was different (vermicompost against sedimentary origin), so it could be that HA function related to oxidative stress or activating peroxidases is a general biological activity feature of many HAs independently of their origin.

AT3G48360.1 encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway. Its biological function is related to the abscisic acid-activated signaling pathway, to the auxin-activated signaling pathway, to the regulation of transcription, DNA-templated, response to carbohydrates, response to cold, response to hydrogen peroxide, response to jasmonic acid, response to nitrate, response to salicylic acid, response to salt stress, response to wounding, response to sugar mediated signaling pathway etc. It seems that although the regulation of this gene is related to a wide range of biological functions, some of the mentioned signaling pathways correlate with previously described physiological SHA functions. The involvement of abscisic acid-activated signaling pathway and the response to abscisic acid could be perfectly connected with the SHA stimulated physiological response of ABA root synthesis linked to $Lp_r$ activation and aquaporin activity involvement (chapter I). Further, the genetic response to auxin-activated signaling pathway and the response to auxin has widely been studied as a response to HA activity (chapter II) and (Canellas et al., 2002; Trevisan et al.,2010; Mora et al., 2010).

We showed that HS treatment repressed the expression of several genes. Remarkably, a large number of them encodes light response processes. These molecular activities have been identified in shoot, but in our case the transcriptomic analyses were carried out with root tissues. Although the molecular behavior of these genes does not seem to be a common feature and should be study further, it is interesting to mention that

the square plates, in which Arabidopsis seeds were germinated, roots were exposed totally to light,. Analyzing specifically the fact that SHA treatment notably repressed the gene expression of light related response compared to control plants, it would be explained by two possible reasons: on the one hand, the possibility that the SHA treatment itself covered up or protected the roots against the light, repressing the expression of these plant genes. Or, as another suggestion, it could be that the activity of SHA in roots counteracted the negative effect that light was generating in roots, repressing the expression of genes that encoded light response. In any case, as proposed by Silva-Navas et al. (2015), light is a stress source for plants roots and influences overall plant growth and responses. So, the use of a square plates growing methodology that prevents illumination to roots in future experiments could generate a better understanding of the matter.

Figure IV, 10 summarizes the physiological response exerted by root SHA application in plant physiology (explained extensively in chapter I and II) linked to the genetic up-regulated response studied in this chapter.

The above discussed preliminary results on the candidate genes involved in the regulation of the response of SHA shows that it is a very interesting tool to study the mechanism of action of SHA. Thus, taking into account that the presented results of RNAseq had some limitations (low gene response differentiation between SHA and control treatments for example and stress source because of light exposure of the whole plant), some new experiments are being carried out. For the future, the experiments on searching on the candidates genes involved in SHA activity will be studied for both the root and shoot and in two parallel ways: first of all the study of the SHA primary effects in the regulation of genes (harvesting the Arabidopsis plants after 1 day of treatment) and also the SHA involved growth related and long distance response in genes (leaving the SHA treatment for a longer period of time).

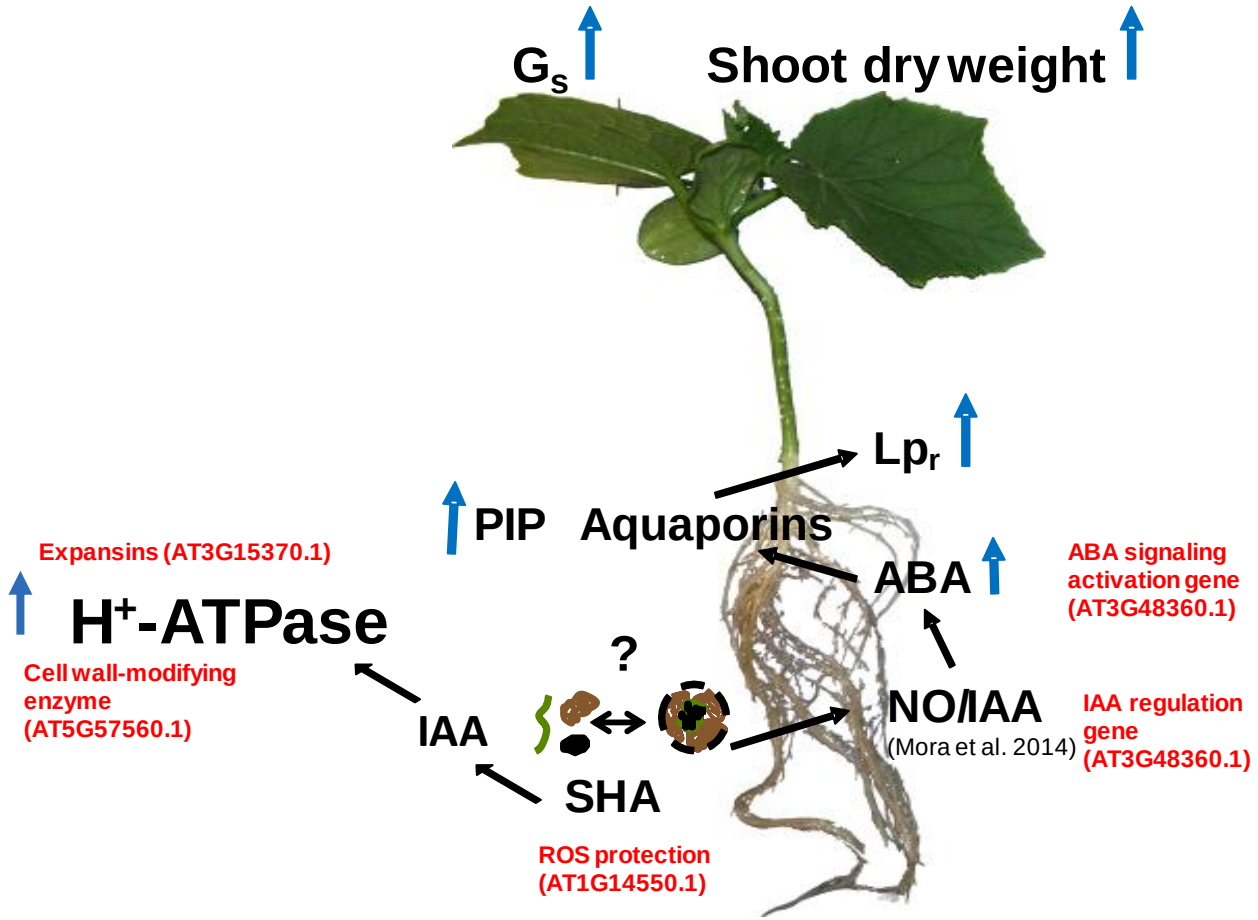


Figure IV, 10: This figure represents the mechanism of action studied in chapter I and chapter II, showing the metabolic or physiologic domains that are modified by SHA root action (in black). The indications in red show the SHA root action up-regulated genes related biological process function (chapter IV).

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## GENERAL DISCUSSION

At present, the mechanism involved in HS action of plant development is scarcely understood and it is not integrated into a holistic view. Therefore, this work focused on carrying out a more in-depth study on the mechanism of SHA action in roots of cucumber plants but relative to the response in the whole plant. Indeed, based on the results obtained for this first objective, this study continued to research the integration of the described facts into the previously reported HAs biological abilities, specifically into the crosstalk with PM-$H^+$-ATPase activity and its involvement in plant root promoting action. Moreover, apart from the study regarding SHA-exerted responses in plant physiology that has been explained, a genetic approach of the SHA response in plant roots was also considered. A final glance at the structural and chemical view of SHA in relation to the obtained biological activity results was carried out.

In relation to the mechanism of SHA action in cucumber plants roots, this study showed that the beneficial effect of SHA on shoot growth was linked to significant increases in both $Lp_r$ and $G_s$ (fig I, 1). These results strongly suggest that the positive effects of SHA application enhance root water uptake. With regard to the mechanisms involved in the positive effect of the application of 100 mg C $L^{-1}$ SHA on root water transport-related parameters, the results showed that the synthesis of root endogenous ABA promoted by SHA plays a relevant role (figure I,3). Further, numerous studies reported the role of root ABA on regulating $Lp_r$ (Hose et al., 2000; Thompson et al., 2007; Tardieu et al., 2010; Sanchez-Romera et al., 2014) and the expression of genes encoding some families of plasma membrane aquaporin proteins (Beaudette et al., 2007; Mahdieh and Mostajeran, 2009). Our experiments indicated that the SHA-mediated increase in $Lp_r$ is also ABA-dependent because the application of Fld (a specific inhibitor of ABA biosynthesis) abolished SHA action on $Lp_r$. In relation to the aquaporin-encoding gene regulation, the obtained data showed that SHA application caused a significant up-regulation of the four *Cs*PIPs studied, showing a different time-course pattern for each one (figure I, 11). With regard to Fld treatment application results, they showed that this treatment caused a significant up-regulation of most of the studied *Cs*PIPs at many harvest times, suggesting that other ABA-independent water uptake regulatory pathways are activated in roots in order to prevent a potential

water deficit stress caused by ABA-functional loss. For the evaluation of SHA exerted gene regulation, two facts need to be taken into consideration: when SHA and Fld were applied together, on the one hand, there was an additive up-regulation for *Cs*PIP 1:2 and *Cs*PIP 2:5 compared to the inhibitory effect (Fld), showing gene expression through an ABA-independent pathway for SHA; on the other hand, there was an effect similar to the one caused by Fld alone for *Cs*PIP 2:1 and *Cs*PIP 2:4, exhibiting SHA ability to enhance *Cs*PIPs gene regulation through an ABA-dependent pathway. In conclusion, the discussed data revealed that the SHA root mechanism for promoting shoot growth in cucumber plants is mediated by ABA-dependent enhancement of $Lp_r$, water uptake and root plasma membrane aquaporin gene expression (not always regulated by ABA).

The described SHA-mediated response in plants is usually described as a typical physiological response of early phase water stress in plants. However, in this study, no stress markers were found, and taking into account that the concentration applied to plants is in the beneficial range, because the final response of SHA reaches the promotion of plant development, more in-depth studies are needed to elucidate if this beneficial theoretical transient stress disappeared with the passage of time or remains with sporadic activation and if this beneficial response is mediated by plant-SHA active interaction.

In order to discriminate the relative role of previously explained SHA-mediated enhancement of root hydraulic conductivity ($Lp_r$) and root PM-$H^+$-ATPase activity in the humic acid promoting effects of shoot and root growths, some experiments were carried out. Our results clearly show that the application of an inhibitor of root PM-$H^+$-ATPase activity (DCC) removed the SHA-mediated increase in both RRGR and SRGR (table II, 2 and table II, 3). These results indicated that, at least under these experimental conditions and in cucumber plants, the enhancement of root PM-$H^+$-ATPase activity caused by SHA appears to play a very relevant role in the root and shoot promoting actions of SHA. However, $Lp_r$ activity appears to be involved in SHA-mediated RRGR and SRGR response because the application of DCC in control and SHA-treated plants also caused a significant decrease in $Lp_r$ values (figure II, 3), although it declined after 72 hours (specifically for DCC+SHA treated plants). In this regard, there

are some studies describing the possible functional relationship between $Lp_r$ regulation and root $H^+$-ATPase activity, principally in plants subjected to abiotic stress such as cold (Lee et al., 2004) or salinity (López-Pérez et al., 2009). The results with the experiments carried out with DCC showed that both parameters appeared to be involved in SRGR and RRGR. However, interestingly, the application of blockers of $Lp_r$, PEG and PAA, removed the promoting action of SHA on $Lp_r$ and SRGR, but did not affect RRGR and significantly increased PM-$H^+$-ATPase activity. This fact indicates that the SHA-mediated enhancement of root PM-$H^+$-ATPase activity might play a secondary role in the improvement of shoot growth caused by SHA, making SHA-mediated $Lp_r$ activity a more relevant parameter.

With regard to root growth however, both PEG and PAA did not affect RRGR. The principal difference between SHA and PEG or PAA is that whereas SHA increased both $Lp_r$ and root PM-$H^+$-ATPase activity, PEG and PAA increased root PM-$H^+$-ATPase activity but decreased $Lp_r$. In relation to plant development, PEG and PAA significantly decreased SRGR (table II, 5) compared to control plants but there were no significant differences on RRGR (table II, 4) compared to control treatment. Therefore, these results suggest that root PM-$H^+$-ATPase activity is involved in the promoting action of SHA on RRGR. However, this effect supports a relevant role of the PM-$H^+$-ATPase dependent pathway in the root promoting effect of SHA. $Lp_r$ dependency cannot be ruled out since DCC and (DCC+SHA) treatments also reduced $Lp_r$ (figure II, 3). In order to discriminate the relative role of each of the SHA-mediated events in RRGR, taking into account that PEG and PAA reduced $Lp_r$ without affecting RRGR and (PEG+SHA) could not recover RRGR (table II, 6), our results indicate that the stimulation of root PM-$H^+$-ATPase activity probably plays a more relevant role than the increase of $Lp_r$ in the mechanism of action of SHA on plant root growth.

Apart from the aforementioned discussion regarding SHA response in plant biology related event, another objective of this work was to study the genetic response of SHA in roots.

Among the different SHA-activated genes, some of the up-regulated responses could be related with part of the mechanism of action described in previous chapters or with

the effects that were previously reported by other authors. AT3G15370.1 gene regulation, identified as a member of an Alpha-Expansin Gene Family, is related to the loosening of the plant cell wall, plant cell wall organization and with the unidimensional cell growth. This gene up-regulation, together with AT5G57560.1 gene that encodes a cell wall-modifying enzyme, could be correlated with cell enlargement as a response of SHA that exerts root growth promotion. In relation to the results obtained in chapter II, it appears that the main SHA-exerted physiological response that promotes root growth promotion is $H^+$-ATPase activity mediated by root IAA synthesis. Along this line of thought, AT2G14960.1 gene was identified as another gene that encodes a protein similar to IAA-amino synthases. Some IAA–amino synthetases maintain auxin homeostasis by conjugating excess IAA to amino acids (Ding et al., 2008). Linked to the root IAA-mediated $H^+$-ATPase activation by SHA, the up-regulation of these genes possibly contributes to control SHA-enhanced root-IAA accumulation homeostasis.

AT1G61750.1 was another up-regulated gene. It encodes a receptor-like protein kinase-related family protein. There are some recent studies that show the potential role of HA from vermicompost on eliciting signaling dependent on $Ca^{+2}$-dependent protein kinase (CDPK) at early stages of lateral plant root development (Ramos et al., 2015).

In relation to AT1G14550.1 gene activation, which encodes a peroxidase superfamily protein, it was previously shown that HA of vermicompost induced peroxidase enzymatic activity in rice plants, leading to a reduction in $H_2O_2$ content and ensuring a greater conservation of membrane permeability and preventing oxidative stress (Calderín García et al., 2012). The involvement of AT1G14550.1 gene in SHA response also corroborates the previously shown SHA-exerted effects in peroxidase activity. Indeed, it is interesting to observe that the origin of the applied HA in both studies is different (vermicompost versus sedimentary origin), so it could be said that the HA function against oxidative stress or activating peroxidases is possibly a general biological activity feature of HAs.

AT3G48360.1 encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway. Its biological function is related to the abscisic acid-activated signaling pathway, to the auxin-activated signaling pathway, related to the regulation of transcription, DNA-templated, response to abscisic acid, response to auxin, response to carbohydrates, response to cold, response to hydrogen peroxide, response to jasmonic acid, response to nitrate, response to salicylic acid, response to salt stress, response to wounding, response to sugar mediated signaling pathway etc. It appears that although the regulation of this gene is related to a wide range of biological functions, there are some mentioned signaling pathways that are correlated with previously described physiological SHA exerted functions. The involvement of abscisic acid-activated signaling pathway and the response to abscisic acid could be perfectly connected with the SHA-stimulated physiological response of ABA root synthesis linked to $Lp_r$ activation and aquaporin activity involvement (chapter I). Furthermore, the genetic response to auxin-activated signaling pathway and the response to auxin has been widely studied as a response to HA activity (Canellas et al., 2002; Trevisan et al.,2010; Mora et al., 2010).

Although the up-regulated genes can be linked to some previously described SHA effects, the repressed genetic response arouses some methodology-related suggestions. In fact, there is quite big quantity of genes that encode light response processes. Along this line of thought, it is interesting to mention that the square plates, in which Arabidopsis seeds were grown, were totally exposed to light, both root and shoot. This light process-related gene response can be explained in two ways: the possibility that the SHA treatment itself covered up the roots against the light (because of the color of the SHA solution) or that the SHA activity in roots counteracted the negative effect that light was generating in root tissues. In any case, as Silva-Navas et al. (2015) have proposed, light can be a stress source in plants roots and it influences overall plant growth and responses, so the use of square plates methodology that prevents illumination to roots in future experiments could generate a better understanding of the matter.

The aforementioned discussion regarding preliminary results on the candidate genes involved in the regulation of the response of SHA shows that it is a very interesting tool

for studying the mechanism of action of SHA. Some new experiments are being carried out along this line of thought. The experiments searching for candidate genes involved in SHA activity will be studied in shoot and root gene response in two parallel ways: the study of the SHA primary effects in the regulation of genes (harvesting the Arabidopsis plants after 1 day of treatment) and also, the SHA-involved growth-related and long distance response in genes (leaving the SHA treatment for a longer period of time).

Finally, as a last objective, once the SHA-exerted biological response was studied, we were encouraged to study the relationship between SHA biological response with SHA chemical and structural features. With the aim of studying HS composition, different fractionation processes and a wide range of chemical characterization techniques have been applied to HS. Among the different chemical or physical nature fractionation methods applied to HS, a chemical fractionation for a stepwise removal of molecules from complex humic matrices has been described by Nebbioso and Piccolo, 2011. Indeed, connected with the fractionation processes, some characterization techniques have been applied to HS in order to elucidate the chemical structure. Parallelly, a structural theoretical model by molecular mechanics was proposed for SHA. The proposed model showed that in the bulk SHA structure, the theoretical particle size measurement of three sub-units fixed the value at 5.64 nm (figure III, 6) which could be correlated with DLS measurement (figure III, 3). Furthermore, the particle size of the domain formed by six sub-units was theoretically fixed at 10.90 nm (figure III, 7) which, although not precise, could also correspond with the other peak shown by DLS (figure III, 3).

In relation to the gravimetric results reached by the humeomic fractionation process (table III, 1), this process yielded a substantial amount of ORG 1 fraction, corresponding to 62.5% of the total bulk humic acid. Some of these aggregates could be linked with fragile molecular forces and therefore could be extracted with a mixture of organic solvents corresponding to 62.5% of the supramolecular nature ORG 1 fraction. The chemical nature of this fraction based on GC-MS spectra (although the characterization was not concluded) was principally hydrophobic and composed of fatty acid (figure III, 10). As for the rest fractionation process, it should be pointed out

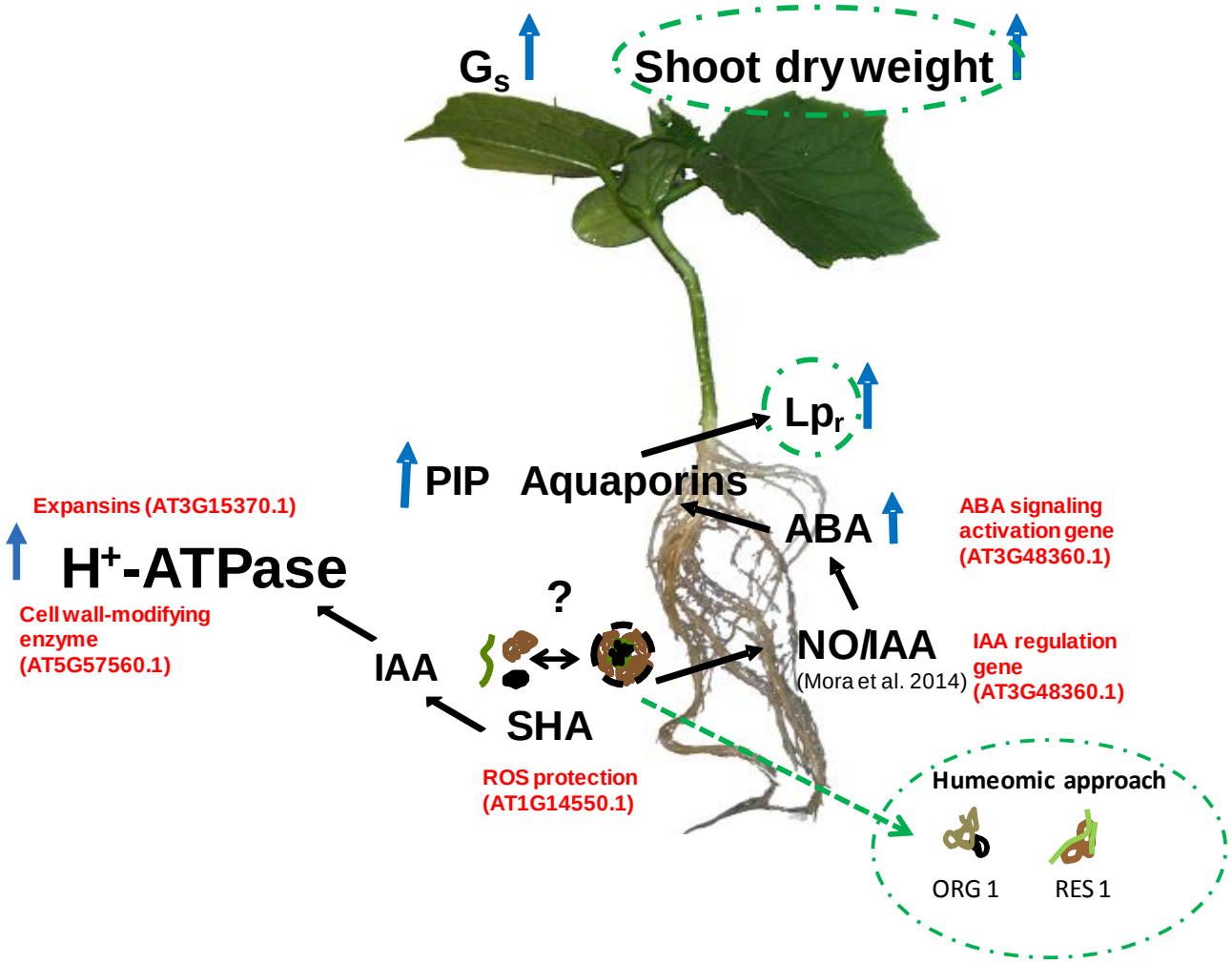
that the final residue (RES 4) yielded close to 10% of the initial amount of SHA and that ORG 2 and ORG 3 fraction yielded nearly the same quantity of material, leaving little fractions quantity-wise for the AQU fractions (table III, 2).

The nature of the rest of the ORG fractions (ORG 2 and ORG 3) was identified as being very similar to ORG 1, showing the existence of a variety of fatty acids (figure III, 12 and 13). Ignoring the analysis of the AQU fractions because they are minor in quantity compared with the other fractions, the last residue (RES 4) yielded close to 10% of the total amount of matter (table III, 2) and it was analyzed by $^{13}$C-NMR technique (figure III, 14). It can be concluded that it is the most aromatic fraction because aliphatic/aromatic ratios for the proposed fractions showed an evolution of increasing aromaticity between the final residue (RES 4) and the previously extracted fractions (ORG 1 until AQU 4) corresponding to 0.664 versus 1.076 measured values (table III, 6).

With regard to physiological activity showed by the extracted fractions, it can be observed that only one of the two fractions (RES 1) was able to increase activity on $Lp_r$ at 72 hours of treatment (figure III, 17). Indeed, it showed a significant increase not only compared with control plant but also compared with the bulk SHA treatment. In line with previous results (Olaetxea et al., 2015, chapter I), this increase in $Lp_r$ caused by RES 1 also showed an increase in SRGR and RRGR (table III, 7 and table III, 8). For ORG 1 fraction, the response in roots was similar to SHA bulk treatment, showing a significant increase compared with control plants (table III, 8) but without showing any increase either in $Lp_r$ or in SRGR (figure III, 17 and table III, 7). With regard to the chemical nature of both fractions, RES 1 fraction showed higher aromatic character whereas ORG 1 fraction was more aliphatic (figure III, 15 and figure III, 16). These results suggest that the promotion shoot growth is likely more dependent on aromatic-related features, while root promotion is sensitive to both aliphatic and, more intensely, aromatic features of SHA.

As a general reflection, the SHA exerted activity on plant biology under short time periods was carried out in this study. The SHA exerted response under longer periods of application will be something interesting to consider for future. In fact, when HAs are applied in agriculture as a source of biostimulants for plants, although at first HA

treated plants in field show benefits in development, shortly after this first response, plants that received HAs as amendement and plants that did not, start to equalize the plant development, although HAs treated plants always show a stronger metabolism to support setbacks (abiotic, biotic or meterologicals, for example). Revising the mechanism of action studied in this work that SHA exerted, some SHA-enhanced growth related activities were described in detail ($H^+$-ATPase activation, IAA-root biosynthesis increase, $Lp_r$ activation, aquaporin activity, genes encoding for expansins etc.). Although there are some more factors acting in field that is necessary to consider, these SHA promoted growth responses could partially explain the SHA produced mechanism of plant development also in field. Moreover, in our experiments, there was a very interesting parameter enhaced by SHA application that could be related with the effect that SHA usually show in plant protection against setbacks: the activation of ROS related gene function, a peroxidase superfamily protein that prevents oxidative stress. In fact, it was previously described by other authors (Calderín García et al., 2012) that the application of vermicompost, which contains HA, induced peroxidase enzymatic activity, leading to a reduction in $H_2O_2$ content and ensuring a greater permability and preventing oxidative stress. In this line, apart from that gene up-regulation, no more stress markers or stress realted activities have been found in our experiments. However, it could be possible that the application of SHA, exerted a kind of eustress , a stress not causing oxidative damage but rather promoting health and growth that involves a mild stress event and the organism´s response to it is usually acclimative (Hideg et al., 2013). This eustres could be an explanation to understand the first response to the prompt plant development exerted by SHA followed by the acclimatation period and at the same time the plant methabolism protection showed by SHA in fields. Nevertheless, this hypothesis or possible explanation needs to be studied more in depth.




This final figure represents the SHA exerted biological mechanism of action, integrating the parameters studied such as, root-ABA synthesis, *Cs*PIPs regulation and $Lp_r$ activity (in chapter I) together with previously reported involvement of NO/IAA signaling pathways (Mora et al., 2014a, 2014b). Furthermore, the studies carried out in discriminating the role of $Lp_r$ and $H^+$-ATPase activity on plant development are also represented (chapter II). In red, SHA action on root gene regulation in Arabidopsis and the plant correlation with plant biological processes (chapter IV). Finally, in green, the humeomic approach carried out for SHA and the biological parameters affected after the application of the fractions.

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# GENERAL CONCLUSIONS

1. In the present study the application of a concentration of 100 ppm of C of a sedimentary humic acid (SHA) increased cucumber plant shoot growth. The beneficial effect of SHA on shoot was accompanied by significant increases in both hydraulic conductivity ($Lp_r$), stomatal conductance ($G_s$), root-ABA (abscisic acid) concentration but not in the concentration of ABA in leaves.

2. The experiments indicated that the SHA-mediated increase in $Lp_r$ was root-ABA dependent. In relation to root plasma membrane aquaporins activity (*Cs*PIPs), SHA application caused significant up-regulation of the four *Cs*PIPs studied. *Cs*PIP 1:2 and *Cs*PIP 2:5 SHA mediated up-regulation was compatible through an ABA-independent pathway while *Cs*PIP 2:1 and *Cs*PIP 2:4 were compatible through an ABA-dependent pathway and both represented the most relevant *Cs*PIPs (in quantity) in cucumber roots.

3. Our results indicated that SHA-mediated enhancement on root PM-$H^+$-ATPase and $Lp_r$ are influenced each other.

4. SHA-mediated enhancement of root PM-$H^+$-ATPase activity might play a secondary role in the improvement of shoot growth caused by SHA, with the SHA-mediated enhancement of $Lp_r$ being more relevant. However, our results indicated that the stimulation of root PM-$H^+$-ATPase activity caused by SHA plays a more relevant role than the increase of $Lp_r$ in the mechanism of action of SHA on plant root growth.

5. As for the operational humeomic-based fractionation method yielded a substantial amount of ORG 1 fraction, corresponding to the 62.5 % of the total bulk SHA. Based on humeomics framework, this fact suggests that ORG 1 probably involves a mixture of aliphatic molecules associated with a more aromatic core (RES 1).

6. With regard to the physiological activity showed by the extracted fractions, it is noticeable that only the fraction RES 1 was able to increase $Lp_r$, SRGR and RRGR significantly compared to both control and bulk SHA treated plants. For ORG 1 fraction, the response in root growth was similar to SHA bulk treatment and did not show increase in SRGR and $Lp_r$. These results showed that the promotion of shoot growth is likely more dependent aromatic-related features, while root promotion is sensitive to both aliphatic and more intensely, aromatic features of SHA.

7. As for the examination of the SHA exerted root-gene regulation, AT3G15370.1 gene up-regulation, which encodes an Alpha-Expansin gene family (related with plant cell wall loosening, plant cell wall organization and with undimensional cell growth), together with AT5G57560.1 gene up-regulation, which encodes a cell wall-modifying enzyme, could be related with SHA showed ability to promote
   root growth. Preliminary results.

8. AT3G48360.1 gene up-regulation, which encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway and its biological function is related with ABA signaling pathway, to the auxin-activated signaling pathway, to the response to auxin and ABA among others, could be correlated with SHA-mediated involvement in ABA and auxin signaling pathways and reponse. Preliminary results.

9. This work contributes to a better knowledge of the mechanism of action of a humic acid from sedimentary origin on plant development.

# ANNEX

Germination process and hydroponic culture conditions

*Cucumis sativus*, L. cv. Ashley sedes were first of all germinated in a germination chamber (GROW-AGP/HR model) for 7 days in darkness at 24º C temperature and 85% relative humidity, on top of perlite and filter paper, moistened with $CaSO_4$ 1mM solution.

Figure A.1: *Cucumis sativus* germinated seeds

Cucumber seedling were transferred to hydroponic culture system in a growth chamber at 25/21 ºC, 70-75% relative humidity and with 15/9 h day/night photoperiod (irradiance: 250 µmol $m^{-2}$ $s^{-1}$) and 400 ppm of $CO_2$ content. Seedling were introduced in some foam rubber sheets (using small cillinders to hold the seedlings), leaving the roots under the sheet and the shoot on top of it. The sheets were placed over the nutritive solution contained in 8L opaque containers. Aeroponic system was built using a central circular tubes system that provided the same air pressure and oxygen to all the containers and roots.

After ten days growing in hydroponic conditions, plants were treated with the specific treatment planned for the experiment. Harvests were conducted at the same time of

the day to exclude diurnal variations, this meant 6 hours after the start of the light period (11 am). The first harvest was conducted after 4 hours of the treatment (treatment at 7 am, harvest at 11 am) and respecting the six hours of the start of light period. The rest of the harvest were conducted after 24, 48 and 72 hours of treatment (applying the treatments to the new containers few minutes before the 11 am).

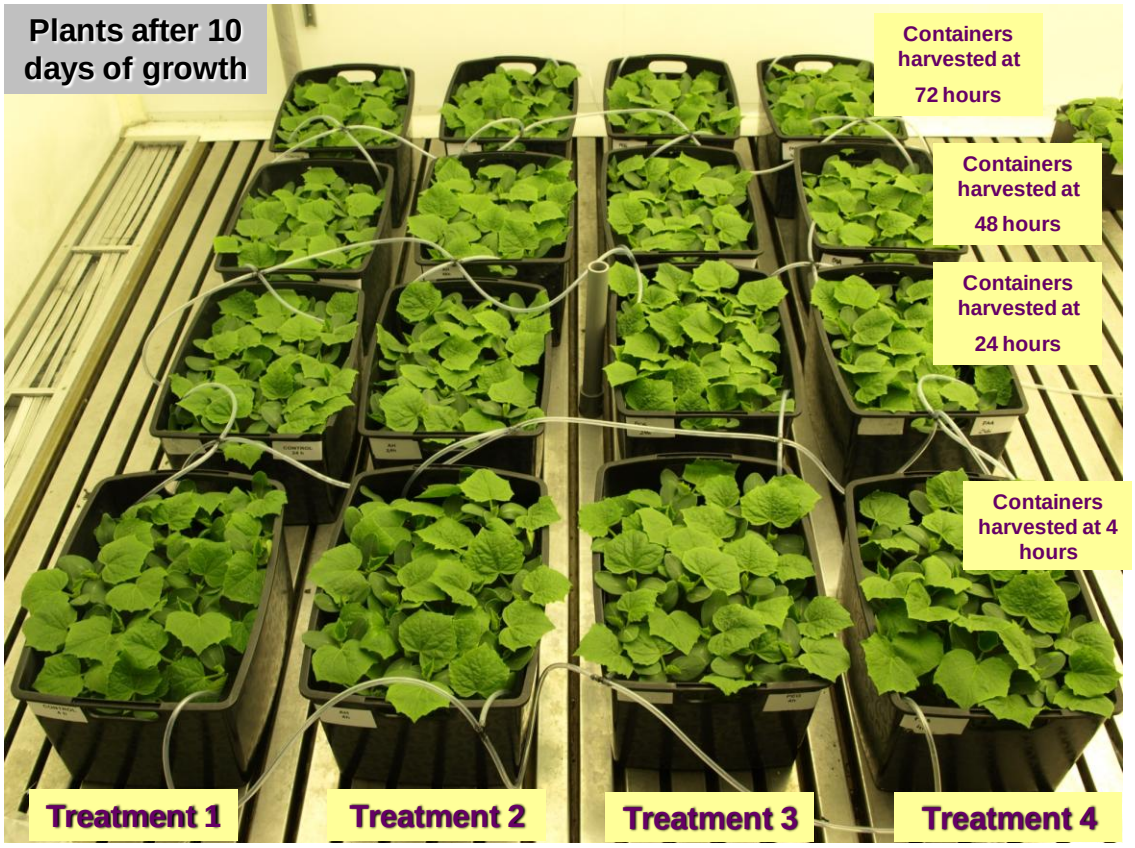


Figure A. 2: Cucumber plants grown in hydroponic system. Experimental model carried out to develop the experiments.

Light system inside the growth chamber was composed by fluorescent and incandescent light sources. The lamps were placed in three different phases in order to provide different light intensity during the whole light period and optimal chromatic quality for plant growing.