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BIOSTIMULANT EFFECTS OF DIFFERENT PRODUCTS ON THE METABOLISM OF AGRO-FOOD PLANTS

EFFETTO BIOSTIMOLANTE DI DIVERSI PRODOTTI SUL METABOLISMO DI PIANTE DI INTERESSE AGRARIO

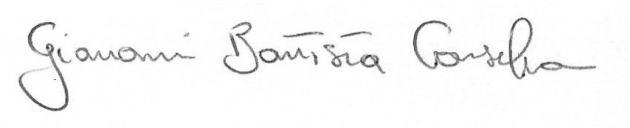
Coordinator: Prof. Stefano Schiavon

Supervisor: Dott. Paolo Carletti

Ph.D. student: Giovanni Battista Conselvan

Declaration

I declare that this thesis has not previously been submitted as an exercise for a degree at the University of Padova, or any other university, and I further declare that the work embodied in it is my own.



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Abstract

Sustainable and environmentally friendly agriculture represents the main strategy to meet rising food needs and to contribute to a stable planet. To achieve these goals, one of the most promising solutions is the adoption of plant biostimulants. Biostimulants are natural compounds which, at low concentrations, are able to enhance plant nutrient uptake, nutrient efficiency, tolerance to abiotic/biotic stresses, and crop yield. Humic substances (HS) fall within the biostimulants definition, as they have positive effects on the uptake of macro and micronutrients that considerably improve the metabolism, the growth and yields of relevant agricultural crops.

With this background, the aims of this Ph.D. project were to i) evaluate metabolic and biochemical status of plants treated with HS to elucidate the action mechanisms of plant biostimulants. This might permit the development of a second generation of biostimulants where synergies and complementary mechanism can be functionally designed. ii) investigate potential biostimulant effects of a fungicide compound.

In manuscript I *Arabidopsis thaliana* plants were treated with HS for eight hours. Root proteins were studied by MS spectrometry coupled with iTRAQ (Isobaric Tag for Relative and Absolute Quantification) technique. 902 protein spots were identified for HS treated roots, where 291 proteins were differentially expressed. Bioinformatic tools such as DAVID, KEGG, IIS and Cytoscape were used to interpret the biological function, pathway analysis and visualization of the network amongst the identified proteins. From this analysis, it was possible to evaluate that most of the differentially expressed proteins were functionally classified into response to inorganic substances, redox homeostasis, energy metabolism, protein synthesis, cell trafficking and division. With the present study an overview of the metabolic pathways most modified by HS biological activity is provided. Moreover, from the analysis of interactomes and DAVID clusters it was possible to observe previously undiscovered HS effects, i.e. on the Ubiquitin and RACK1A interactome subnetworks.

In manuscript II *Arabidopsis thaliana* plants, grown in hydroponic conditions, were treated for 8 h with indole-3-acetic acid (IAA), HS from International Humic Substances Society (IHSS) and HS from earthworm *faeces* (EF), respectively. Humic substances structural characteristics were analysed by ^{1}H NMR an FT-IR spectroscopies. Root and leaf free amino acids, sugar alcohols and carbohydrates contents, and leaf amino acids from protein hydrolysis were identified and quantify by gas chromatography-mass spectrometry (GC/MS), and liquid

chromatography-mass spectrometry (LC/MS) techniques. Canonical discriminant analysis (CDA) was used to evaluate the influence of the treatments on the studied parameters. EF treatment had the highest influence on metabolites profiles compared to the control, IAA and IHSS. CDA analysis highlighted a clear distinction between EF and IHSS plant physiological responses, depending on the different chemical and structural properties of the HS. IAA-treated plants resulted not significant different from the control. A better understanding of the specific effects of different HS, also related to their chemical characteristics, might serve as a basis for the identification of marker compounds for HS bioactivity.

In manuscript III the biostimulant activity of HS extracted from four leonardites is analysed on maize seedlings. After 48 h of treatment with five concentrations (0, 0.1, 0.5, 1, and 10 mg C L−1) of HS, root growth and morphology, glutamine synthetase (GS) activity, glutamate synthase (GOGAT) activity, total protein content, soluble sugars content, phenylalanine ammonia-lyase (PAL) activity, soluble phenols, and free phenolic acids were analyzed. HS from different leonardites had similar spectroscopic pattern, with small differences. HS_USA best enhanced total root growth, root surface area, and proliferation of secondary roots. Plant nutrient use efficiency was enhanced by HS_4, HS_USA and HS_B, with increment of GS and GOGAT enzymes activity and total protein production. HS stimulated also PAL enzyme activity, followed by a higher production of total soluble phenols. This study found that, although the activity of the HS depended on the origin of the leonardite, these compounds can be attributed to the biostimulant products, eliciting plant growth, nitrogen metabolism, and accumulation of phenolic substances.

In the manuscript IV it was investigated the potential biostimulant side-effects of sedaxane. Physiological changes in disease-free maize seedlings at increasing application doses (25, 75 and 150 μg a.i. seed^{-1}) under controlled sterilised conditions were analysed. Sedaxane had significant auxin-like and gibberellin-like effects, with maximum benefits attained at the intermediate dose. Root length (+60% *vs*. untreated controls), area (+45%) and forks (+51%) were significantly increased. Sedaxane enhanced leaf and root glutamine synthetase (GS) activity resulting in greater protein accumulation. Sedaxane also improved leaf phenylalanine ammonia-lyase (PAL) activity, which may be responsible for the increase in shoot antioxidant activity (phenolic acids). It is concluded that, in addition to its protective effect, sedaxane can facilitate root establishment and intensify nitrogen and phenylpropanoid metabolism in young

maize plants, and may be beneficial in overcoming biotic and abiotic stresses in early growth stages.

As a general conclusion, even if HS were extracted from different sources were applied on different plant species, they displayed similar biological activities. Proteomics and metabolomics studies confirmed that "–omics" techniques are essential tools to have a 'panoramic' view on metabolic changes happening inside an organism after a positive or negative external perturbation.

List of the Manuscripts

This Ph.D. Project is composed by the following four Manuscripts (the manuscripts are named "Manuscript" followed by roman number in the text).

Manuscript I — Antonio Masi, Giovanni Battista Conselvan, Micaela Pivato, Giorgio Arrigoni, Tayyaba Yasmin, Sohaib Roomi, Paolo Carletti (2017). Protein profiling of Arabidopsis roots treated with humic substances. (to be submitted to Plant and Soil)

Manuscript II — Giovanni Battista Conselvan, David Fuentes, Cristina Peggion, Ornella Francioso, Andrew Merchant, Paolo Carletti (2017). Effects of humic substances and indole-3-acetic acid on Arabidopsis sugar and amino acid metabolic profile. (under revision to Plant and Soil)

Manuscript III — Giovanni Battista Conselvan, Diego Pizzeghello, Ornella Francioso, Michele Di Foggia, Serenella Nardi, Paolo Carletti (2017). Biostimulant activity of humic substances extracted from leonardites. Plant Soil. doi: 10.1007/s11104-017-3373-z

Manuscript IV — Dal Cortivo Cristian, Giovanni Battista Conselvan, Paolo Carletti, Giuseppe Barion, Luca Sella, Teofilo Vamerali (2017). Biostimulant effects of seed-applied sedaxane fungicide: morphological and physiological changes in maize seedlings. Front Plant Sci. https://doi.org/10.3389/fpls.2017.02072

List of other Contributions

The other Contributions (chronologically listed in the follow table) represent the writing production (Abstract of Poster/Abstract of Oral Presentation) related to attendance as author to international conferences and disseminating scientific contributions.

Contribution I	Proteomic profiling of Arabidopsis roots treated with humic substances – Abstract and poster Micaela Pivato, Giorgio Arrigoni, Giovanni Battista Conselvan, Serenella Nardi, Sohaib Roomi, Antonio Masi, Paolo Carletti. Congresso ItPA, 23-28 giugno 2015, Milano.
Contribution II	Suoli sottoposti ad agricoltura conservative: studio dei parametric chimico-biologici – Abstract and poster Conselvan G. B., Pinto S., Nardi S., Furlan L., Chiarini F., Menta C., Carletti P. XXXIII convegno SICA, X convegno IHSS – Italian Chapter, 16-18 settembre 2015, Bologna.
Contribution III	Network analysis of differentially expressed proteins in Arabidopsis roots treated with humic substances – Abstract and oral presentation Carletti P., Conselvan G.B., Pivato M., Arrigoni G., Roomi S., Masi A., Nardi S. XXXIII convegno SICA, X convegno IHSS – Italian Chapter, 16-18 settembre 2015, Bologna
Contribution IV	The biostimulant activity of humic substances: a proteomic approach – Abstract and poster Conselvan G. B., Pivato M., Arrigoni G., Roomi S., Masi A., Nardi S., Carletti P. 1st DAFNAE postgraduate scientists meeting Legnaro, 22-23 settembre 2016.

1 Introduction

1.1 Plant biostimulants

Last decade mankind entered in a new geological epoch, the Anthropocene, where humans are the largest driver of planetary changes (Steffen et al. 2015). To achieve human development at all scales (from local farms to cities, nations and the world), global sustainability is considered to be a fundamental prerequisite (Folke et al. 2005). By 2050 the global population will grow from seven to nine billion people (Godfray et al. 2010), requiring an increase in global food production of between 60 and 110 % (Pardey et al. 2014). In this 'event', agriculture plays a crucial role; it is considered the world's largest driver of global environment change, and also the most affected by these changes (Field et al. 2014). To meet rising food needs and to contribute to a stable planet, reducing environmental impacts, sustainable agriculture is the only strategy (Rockström et al. 2017). This could be achieved following some important policies: (1) improving efficiency in the use of resources; (2) applying direct actions to conserve, protect and enhance natural resources; (3) protecting and improving rural livelihood, equity and social well-being; (4) enhancing resilience of people, communities and ecosystems; (5) applying responsible and effective governance mechanisms (FAO 2014).

To actualize these strategies, one of the most innovative solutions is the use of plant biostimulants. Biostimulants are compounds that *"contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality"* (EBIC 2017). The definition of biostimulant is not univocal, because it may be a substance (single chemical compound or a group of compounds with a well-known established origin) or a microorganism (du Jardin 2015). Biostimulants are considered as borderline substances between fertilizers and plant protection products, because they do not contain any nutrient for plants, and do not give direct protection against pest (du Jardin 2015; La Torre et al. 2016).

Because of a lack of formal definition, the regulatory situation of biostimulants is very complex. In EU biostimulants could be placed on the market following the Regulation EC No 2003/2003 on fertilizers or the Regulation EC No 1107/2009 on plant protection products (du Jardin 2015).

Despite this regulatory situation, the global market of biostimulants is continuously growing; in 2021 it is expected to reach $ 2.91 billion in sales, with an annual growth rate of 10.4 %. In terms of area of application, the biostimulant market will reach 24.9 million hectares by 2021, with an annual growth rate of 11.7%. In 2015 Europe accounted for the largest market, while Asia-Pacific region is the fast-growing market from 2016 to 2021 (Biostimulant market 2016). The main drivers of this market are: (1) growing importance for organic products; (2) increase of application of biostimulants in developing countries; (3) acceptance among customers of these products, as they satisfy specific crop needs (Povero et al. 2016).

1.2 Biostimulant categories

International organizations and scientists recognized six main categories of biostimulants: protein hydrolysates and amino acids, seaweed extracts, chitosan, inorganic compounds, microbial inoculants and humic substances (HS) (Calvo et al. 2014; du Jardin 2015; EBIC 2017).

Protein hydrolysates and amino acids

Proteins hydrolysates consist of a mixture of peptides and amino acids with animal or plant origin. The individual amino acids include both the twenty structural and non-protein amino acids. These products are usually obtained by chemical, thermal or enzymatic hydrolysis of agroindustrial by-products, from plant (crop) or animal (epithelial or connective tissues) residues (Calvo et al. 2014; du Jardin 2015).

Protein hydrolysates and amino acids have multiple effects on treated plants. In maize, alfalfa hydrolyzates enhanced N uptake and assimilation, regulating the activity of enzymes involved in N metabolism (NADH-dependent glutamate dehydrogenase, nitrate reductase, nitrite reductase glutamine synthetase and glutamate synthase) and the gene expression of nitrate reductase (Ertani et al. 2013; Schiavon et al. 2008). In the same plants, these hydrolysates stimulated the activity of enzymes of TCA cycle, suggesting that there was a cross talk between C and N metabolism. Exogenous application of amino acids played a signaling role in regulating nitrogen adsorption by roots (Miller et al. 2007). Protein hydrolysates and specific amino acids (e. g. proline and betaine) stimulate plant defenses to biotic and abiotic stress, increasing the tolerance to salinity, drought, temperature and oxidative condition (Ashraf and Foolad 2007; Chen and Murata 2011). When they are applied to soils, protein hydrolysates increase microbial biomass and activity, soil respiration and fertility (du Jardin 2015).

Seaweed extracts

From ancient times seaweed has been used as soil amendment and fertilizer, but only from 1950s seaweed extracts are used as commercial products in agriculture and horticulture (Craigie 2011; Khan et al. 2009). Most of the extracts are from the phylum of brown algae, including *Ascophyllum nodosum, Fucus, Laminaria, Sargassum*, and *Turbinaria* spp. (Sharma et al. 2012). These extracts are a complex mixture of organic and mineral compounds, including unique polysaccharides like lamarin, fucoidan and alginates, and particular hormones which are not present in terrestrial plants (Khan et al. 2009). Concentration of carbon, nitrogen lipid and ash varies significantly from one species to another, and the content of indole-3-acetic acid (IAA) range from 3 to 47 ng/g (Sharma et al. 2012).

Seaweed extract are recognized to have several biostimulant activities. They are known to improve growth and yield in agricultural and horticultural crops. Thanks to the presence of different phytohormones (e.g. auxin and cytokinin), seaweed extracts enhanced root development in several crop species after foliar application. They are known to increase nutrient uptake and the accumulation of macro (N, P, K, Ca, S, Mg) and micronutrients (Zn, Mn, Fe) (Calvo et al. 2014). Seaweed extracts have also an influence on plant metabolism and physiology. Fan et al. (2013) demonstrated that, in treated spinach, brown algal extracts increased the transcription of enzymes involved in N metabolism, antioxidative capacity and glycine betaine synthesis, improving the production of proteins, antioxidant capacity, phenols and flavonoid content. In *Brassica napus* seaweed extracts upregulated the expression of genes involved in carbon fixation, N and S metabolism (Jannin et al. 2013). Moreover, seaweed extracts enhance tolerance to abiotic stress such as drought, salinity and extreme temperatures, and when they are applied to soils they increase microbial activity and diversity (Calvo et al. 2014).

Chitosan

Chitosan is a co-polymer of N-acetyl-D-glucosamide and D-glucosamide, and it derives from the deacetylation of chitin. Thanks to its properties, chitosan is used in several industries, like food, cosmetic, medical and agricultural. In plants chitosan elicits multiple physiological responses binding a wide range of cellular components, such as DNA, plasma membrane, and cell wall constituents, but also binding receptors responsible for defense gene activation (du Jardin 2015; Pichyangkura and Chadchawan 2015). Thanks to these properties, chitosan was extensively studied as biostimulant product on cereals, ornamental, fruit and medicinal crops.

On several vegetables, soil or foliar application of chitosan enhances growth and yield, meanwhile on ornamentals it stimulates the flowering process. It is well known that chitosan increases plant resistance to biotic and abiotic stresses: in treated plants chitosan induced enzyme activity in the Reactive Oxygen Species (ROS) scavenging system, the phenolic compound biosynthesis, and the activity of the nitric oxide pathway. Moreover, chitosan application prolongs fruit shelf life, decreasing the incidence of infection and preventing the weight loss (it reduces the respiration rate) (Pichyangkura and Chadchawan 2015).

Inorganic compounds

Alluminium (Al), cobalt (Co), sodium (Na), selenium (Se) and silicon (Si) are called beneficial elements, because at low concentrations they can promote plant growth and may be essential for particular taxa. It has been reported that these five compounds have beneficial influences on different functions: plant growth, quality of plant products, nutrient uptake and resistance to herbivory, pathogen attacks and abiotic stresses (heavy metals, salinity, drought, extreme temperature and UV radiation) (Pilon-Smits et al. 2009).

Microbial inoculants

Microbial inoculants are bacteria, fungi, or arbuscular mycorrhizal fungi (AMF) which are isolated from different environments such as soil, plants, plants residues, water and composted manures. In the last two decades, the use of microbial inoculants is gradually increasing, not only for their agronomical benefits, but also as a tool to solve numerous environmental problems. It has been well documented that microbial inoculants improve plant nutrient uptake and nutrient status. In several crops (e.g. cotton, wheat, sugarcane and corn) the inoculation of *Azospirillum* spp. increased the nitrogen content. Different microorganisms were recognized to increase the absorption of macro (P and K) and micronutrients enhancing their solubilization, with a consequent indirect induction of root biomass, root surface area, and root hair growth. Microbial inoculants can positively modify plant hormone status through the production or degradation of auxins, cytokinins, gibberellins, and ethylene. These hormones have a direct influence on several physiological processes, such as root initiation, root and shoot elongation, root hair formation, seed germination, floral induction and fruit growth. Moreover, the application of microbial inoculants increase plant tolerance to drought and salinity stress (Calvo et al. 2014).

Humic substances.

They will be deeply described in the following chapter.

1.3 Humic substances

Humic substances (HS) are widely considered as a group of plant biostimulants.

HS are natural organic amorphous mixture of molecules deriving from the chemical degradation and microbial decomposition of plant and animal residue in the soil (a process called humification) (Nardi et al. 2009). Plant lignin, polysaccharides, melanin, cutin, proteins, lipds, nucleic acids are some of the components which are involved in this process. HS are the most abundant component of organic matter on earth, present on both terrestrial and aquatic systems (Nardi et al. 2002).

They have a great influence on soil fertility, participating in several agronomic, environmental, and geochemical processes. Indeed, HS have on the soil (a) physical effects, contributing to the formation and stability of soil structure and porosity; (b) physico-chemical effects, improving nutrient adsorption and availability; (c) chemical effects, participating to chemical reactions (e.g. production of secondary carbonates which contribute to soil carbon sequestration); (d) biological effects, affecting the diversity and biological activity of soil microorganisms (Bronick and Lal 2005; du Jardin 2012; Nardi et al. 2009).

In addition to the effects on soil properties, HS have also an important impact on plant physiology, enhancing crop yield, plant growth, nutrient uptake and resistance to biotic and abiotic stresses (Nardi et al. 2002). HS influence the absorption of macro and micronutrients, improving the metabolism, growth and yield of agricultural crops (Nardi et al. 2009; Puglisi et al. 2009). Thanks to their hormone-like activity, HS induce root architecture changes through the lateral roots and root hair production (Canellas and Olivares 2014; Trevisan et al. 2010). HS have also an influence on primary and secondary metabolism, improving N assimilation, energy and phenol products production (Nardi et al. 2007; Schiavon et al. 2010; Vaccaro et al. 2015).

1.3.1 Structure of humic substances

Many authors think that it is essential to know the composition and structure of HS to better predict and understand their relationship with the soil and their influence on plant physiology and metabolism (Schulten and Leinweber 2000).

The composition of HS was studied by Schulten and Leinweber (2000), where they identified the primary molecular building blocks: aliphatic chains, quinones, phenols and sugar-like groups. These functional groups are responsible for most of the functions attributed to HS.

However, due to the source and the environments where they originated, HS have a high variability in the chemical composition and molecular structure. Thanks to this heterogeneity HS perform ecologically and environmentally vital tasks (MacCarthy 2001).

Nowadays, the supramolecular structure theory of Piccolo (2002) is one of the proposed theories to describe the structure of HS. HS are considered as heterogeneous supramolecular association of small molecules with various origin, which are held together by weak forces such as dispersive hydrophobic interactions (van der Waals, π-π, and CH-π) and hydrogen bonds. Polar groups constitute the external layer, whereas plant macromolecules make up the hydrophobic interior domain. Because the interconnections between molecules are very week, slight changes in the pH will cause humic polymers to fracture in smaller aggregates. The fractured molecules probably reform micelles, associating with other free radicals, metals or impurities. These processes of micellization and disaggregation could continue indefinitely, causing huge changes in the physical makeup of HS (Muscolo et al. 2013; Šmejkalová and Piccolo 2008).

HS can be subdivided into three main fractions: humic acids, fulvic acids and humins. At alkaline pH values humic acids (HA or high-molecular-weight (HMW) fraction) are extracted from soil or another matrix. HA have average molecular weights (MW) from 2000 Da for aquatic materials to greater than 10^6 Da for solid-derived materials, mainly composed by aromatic and aliphatic molecules, where phenol and carboxylic acid functional groups are the most abundant (Nardi et al. 2009). Fulvic acids (FA or low-molecular-weight (LMW) fraction) are extracted at all pH values; they have an average MW of 600-900 Da, and they are mainly composed by carboxyl (COOH) and hydroxyl (COH) groups. The third fraction is composed by humins, which are not soluble at any pH value, are the most resistant fraction to microbial degradation, and are considered the last product of the humification process. Humins present several functional groups in their structure such as esters, methoxy alkanes, polar aromatic groups, and sugar derivatives (de Melo et al. 2016; de Oliveira et al. 2017; Nardi et al. 2009).

1.3.2 Biostimulant activity of humic substances

Several studies reported the beneficial effects of HS on plant growth and physiology.

The most reported and studied effect of HS on plant is the effects on roots. It has been demonstrated that HS have two main type of phenotypic effects on root development: 1) micromorphological effects, increasing hairs and lateral roots proliferation; 2)

macromorphological effects, increasing root dry weight, secondary root number, and root thickness (Canellas and Olivares 2014; Canellas et al. 2002; Mora et al. 2012; Nardi et al. 2009; Trevisan et al. 2010). Many authors reported that these positive influences could be due to an auxin-like activity (Cacco and Dell'Agnola 1984; Nardi et al. 1988; Piccolo et al. 1992). Muscolo et al. (1998) demonstrated that this auxin-like activity was due to the presence of the auxin indole-3-acetic acid (IAA) in HS. More recently also Schiavon et al. (2010) and Trevisan et al. (2010) reported that HS, which induced root growth on tested plants, contained IAA. The hormone-like activity could be also explained by a similar action of HS to IAA. This was demonstrated on carrot cell cultures where LMW humic fraction interacted with cellular membranes on the same way as IAA (Muscolo et al. 2007). Trevisan et al. (2010) demonstrated also that in Arabidopsis lateral root primordia HS activated the auxin synthetic reporter DR5::GUS in a way comparable to exogenously applied auxins. These data were further supported by the enhancement of the transcription of the early auxin responsive gene IAA19. Auxins are one of the most important morphogenic compounds involved in the regulation of plant growth and development. Auxins are best represented by indole-3-acetic acid (IAA), which is produced in the apical meristem of the shoot and can be transported to the roots via phloem. IAA is involved in the root system architecture with a pivotal role in primary root, lateral root and root hair development (Hager 2003). Auxin signals elicit groups of pericycle cells to re-enter the cell cycle and establish lateral root mitotic sites. Auxins also have an influence on the plasma membrane H^+-ATPase activity, which excretes H^+ ions into the cell wall compartment and takes up K^+ ions through an inside rectifying K^+ channel. The auxin-enhanced H^+ pumping lowers the cell wall pH, activates pH-sensitive enzymes and proteins within the wall, and initiates cell-wall loosening and extension growth. (Benfey et al. 2010; Casimiro et al. 2001; Hager 2003). Quaggiotti et al. (2004) reported that low molecular weight humic substances were able to influence H^+-ATPase enzyme activity on maize plants, increasing of two-fold the mRNA levels of the H^+-ATPase isoform MHA2. This study was also confirmed by Canellas et al. (2009) who demonstrated that HA increased root area of treated maize plants due to also an enhancement of ATPase activity in root cells. Moreover, when H^+-ATPase enzyme is activated, it generates the proton motive force necessary for the ion and metabolite transport across the plasma membrane (Morsomme and Boutry 2000). In maize roots treated with HS was observed a higher nitrate uptake related to the stimulation of H^+-ATPase activity (Pinton et al. 1999).

Lateral root development is also stimulated by HS through the influence of nitric oxide (NO) signaling. Root development stimulation and the H^+-ATPase activation elicited by HA or external IAA was found to depend on mechanisms which use NO as messenger (Zandonadi et al. 2007).

Another area of study seeking to understand how HS influence plant growth and development is the role of reactive oxygen species (ROS) (Berbara and García 2014; García et al. 2014; García et al. 2012). ROS signaling is involved in different plant metabolic processes, including regulation and development of plant growth, response to biotic and abiotic stresses, and cell death (Suzuki et al. 2012). In rice plants treated with HA an increment of ROS production and accumulation in roots, with a concomitant increase in root growth was observed (Berbara and García 2014). ROS produced by NADH oxidase enzymes create a Ca^{2+} gradient in the apical root region, leading to secondary root growth (Mori and Schroeder 2004).

The results reported above, demonstrate that there is not a single mechanism responsible for the beneficial effects of HS on plant growth, but there is a complementary and interconnected signaling pathway which include both hormonal networks and secondary messengers (Garcia et al. 2016).

It is widely documented that HS have also influences on primary and secondary metabolism. As described above, HS enhance root growth and development. In these growing cells/organs, there is a higher activity of N metabolism, of respiratory pathway and glycolysis to support the elevated protein synthesis and energy request (Fernie et al. 2004).

N metabolism is the basis of amino acids, proteins, enzymes and nucleotide generation. N is assimilated as nitrate (NO_3^-) or ammonium (NH_4^+) by plant roots (Stitt et al. 2002). The ammonium, derived from nitrate or directly from ammonium uptake, is further assimilated into N organic compounds. Decreasing pH at the root surface, HS facilitate H^+/NO_3^- symport (Nardi et al. 2000) and stimulate nitrate uptake (through H^+-ATPase) and transport in the plant (Quaggiotti et al. 2004). HS also influence the activity of several enzymes involved in the N metabolism. Panuccio et al. (2001) and Ertani et al. (2011) observed that on treated plants HS induced a higher activity of glutamine synthetase (GS; EC 6.3.1.2), glutamate synthase (GOGAT; EC 1.4.7.1), malate dehydrogenase (MDH; EC 1.1.1.37), glutamate dehydrogenase (GDH; EC 1.4.1.3) and phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31). These enzymes are responsible for the incorporation of NH_4^+ into organic compounds.

HS are also reported to influence glycolysis, tricarboxylic acid cycle (TCA) and carbohydrate

metabolism. Nardi et al. (2007) observed that on maize plant, the Fraction III of HS and humic acids (HA) extracted from Fulvudand soil positively influenced the activity of glycolysis enzymes glucokinase (GKC; EC 2.7.1.2), phosphoglucase isomerase (PGI; EC 5.3.1.9), PPi-dependent phosphofructokinase (PPi-PFK; EC 2.7.1.90) and pyruvate kinase (PK; EC 2.7.1.40), and TCA cycle enzymes citrate synthase (CS; EC 2.3.3.1) and malate dehydrogenase (MDH; EC 1.1.1.37). Canellas et al. (2013) reported that maize plants treated with HS had a lower leaf content of free carbohydrates with a reduction by 60 % of glucose, fructose, and starch compared to the control. This suggests that these humic materials positively affect a wide range of physiological processes, requiring an over-working of the respiratory pathway to produce energy for different metabolic pathways (Fernie et al. 2004).

HS have also a strong influence on secondary metabolism. Schiavon et al. (2010) demonstrated that HS enhanced the expression of the phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) and tyrosine ammonia lyase (TAL; EC 4.3.1.23) that catalyse the first main step in the biosynthesis of phenolics. PAL converts phenylalanine to trans-cinnamic acid and TAL tyrosine to p-coumaric acid. The expression of PAL/TAL was accompanied by phenol accumulation in leaves. Also Olivares et al. (2015) reported a significant increase of PAL activity in tomato leaves treated with humates extracted from vermicompost and a decrease of the field incidence of *Phytophora infectans*, and Hernandez et al. (2015) observed similar trends (enhance on PAL activity) in lettuce.

1.3.3 Humic substances extracted from leonardites

HS can be extracted from humified organic matter (e.g. from soil), from compost, vermicompost, or from mineral deposits such as leonardite or coal (du Jardin 2012).

Leonardite is an oxidized form of lignite with a medium-brown coal-like appearance. It is found at shallow depth over more compact coal in various coal mines around the world, mainly in the USA (Fernandez et al. 1996; Stevenson 1979). This brown coal, particularly enriched in humic C (30–80%), is used to manufacture a wide range of commercial HS products.

It was demonstrated that HS from leonardites encode for typical characteristics of HS biostimulants, enhancing plant growth, yield and resistance to biotic and abiotic stresses.

On tomato plants (hybrids Astona and Gloria) under salt stress, the treatment with Humilton® 60S (23.6% humic acid and 1.1% fulvic acid from leonardite) ameliorated the negative consequences of osmotic stress exposure, improving water uptake and total dry weight per

plant. Moreover, the fresh fruit weight increased to 16.6% for Gloria and to 45.3% for Astona (Casierra-Posada et al. 2009). On *Arnica montana,* after two years of application, Powhumus® WSG 85 (HS extracted from leonardite) significantly induced more generative shoot and flower heads with higher diameter in comparison to the controls (Sugier et al. 2013). David et al. (2014) demonstrated that potassium humate salts extracted from lignite, and potassium humate regenerated from lignite with two oxidizing agents (nitric acid and hydrogen peroxide), positively influenced root growth and division, starch and protein contents in treated *Zea mays* seedlings. Low molecular weight fraction of HS extracted from leonardites enhanced growth, leaf width and area index of snap bean seedlings; treated plants had also double root surface area and length compare to the control (Qian et al. 2015). Moreover, in an *in vitro* study, HS from leonardite significantly enhanced primary root growth and number of lateral root of birch shoot explants, by influencing polar auxin transport (PAT), and alcohol dehydrogenase (ADH; EC 1.1.1.1) and glutamine synthetase (GS; EC 6.3.1.2) gene transcript levels in roots (Tahiri et al. 2016; Tahiri et al. 2015).

1.4 New techniques to study biostimulant activity of humic substances

In the plant cell, DNA is continuously transcribed into RNA, which is translated into proteins. Proteins are intrinsic in the maintenance of biochemical pathways which lead to the production of metabolites. In the field of molecular research “omics technologies” have become the new mantra (Debnath et al. 2010) and the most important are genomics, transcriptomics, proteomics and metabolomics. Genomics is the study of the complete set of genetic materials in an organism and it comprises the sequencing and analysis of genes (Bartel 2004). Transcriptomics evaluates the RNA produced by DNA transcription of a cell, tissue or organism at precise time point. Proteomics aims to study the total protein content of a cell or sub-cellular compartment while metabolomics analyses the metabolites (amino acids, lipids, organic acids, or nucleotides and so on) present in a cell or tissue (Davies 2010). Transcriptomics, proteomics and metabolomics are all dynamic domains, as affected by interactions between the organism and external stimuli (Capozzi and Bordoni 2013).

For genomics and transcriptomics, microarray is the most common analysis for gene expression profiling. Microarray uses information created by genome sequencing (www.genomesonline.org) and form several sequences of expressed sequence tag (ESTs),

which provide information on genes expressed in specific cells, tissue and organs (Davies 2010).

For proteomics analysis two techniques are used: two-dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) linked to mass spectrometry for protein identification, and shotgun proteomics. With 2D-PAGE *ca.* 1000-2000 proteins can be separated and detected by gel-gel comparisons (on internet several images of 2D-PAGE separations are available). Alternatively, with shotgun proteomics all proteins are digested into peptides which are analyzed using liquid chromatography–mass spectrometry (LC-MS) techniques (Davies 2010). With mass spectrometer, it is possible to enhance quantitative accuracy by identifying and quantifying many peptides for each protein in a single experiment (Larance and Lamond 2015). Three methodologies are mainly used for the relative quantification of samples: (1) label-free quantification (protein quantification by using data derived from spectra of MS/MS, number of peptides identified and intensity of each peptide observed) (Cox et al. 2014); (2) *in vivo* metabolic stable labeling (isotope label is introduced into every protein during cell growth and division, which generates a labeled standard for every protein in the sample) (Bantscheff et al. 2012); (3) *in vitro* stable-isotope labelling (tandem mass tags (TMTs) and isobaric tags for absolute and relative quantification (iTRAQ) are the two most popular chemical labelling methods which target primary amides of peptides using stable-isotopes labels) (Bantscheff et al. 2012).

A wide range of technologies are used for metabolomics, based on spectrometry and high-resolution chromatography linked to different detection methods (mainly mass spectrometry). Most of the instruments used for metabolomics analyses are Nuclear Magnetic Resonance (NMR) spectrometry, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (Davies 2010).

Datasets generated from -omics experiments are usually a large list of genes, proteins or metabolites identifications. To give a biological meaning to these large datasets, several Bioinformatics tools have been developed. Gene Ontology is used for the functional annotation (molecular function, biological process and cellular component) of the identified molecules (Ashburner et al. 2001). Once molecules are functionally annotated, other tools could be applied for the identification of functional patterns and overrepresentation of biological functions or processes present in the –omics dataset (e.g. DAVID (Huang et al. 2009), KEGG (Kanehisa et al. 2016), geneMANIA (Warde-Farley et al. 2010), IIS (Carazzolle et al. 2014).

Finally, visualization tools largely contribute to illustrate and study molecule-molecule interactions and cellular organizations (e.g. Cytoscape (Shannon et al. 2003)).

In the last decade, these new molecular "omics" approaches have been used to characterize the complex network of mechanisms responsible for the beneficial effects of HS on plant metabolism. In 2011, Trevisan et al. applied a transcriptomic approach based on the detection of cDNA-AFLP markers to identify the genes potentially involved in the regulation of the response to HS in *Arabidopsis thaliana*. 133 transcript-derived fragments were identified to be differentially expressed after HS treatment, where 75% were up-regulated and 25% down-regulated compared to the control. Using BlastGO (Conesa et al. 2005) software 94 of 133 detected sequences were functionally annotated. The majority of the annotations were attributed to 'metabolic process' and 'cellular process' confirming that HS treatments could affect primary metabolism acting on gene transcription. Most of the genes were expressed at the seedling developmental stage, in particular in root tip, where seem to target vesicle trafficking, transport mechanism, gene transcription, protein regulation and Ca_2^+ signalling. In *Brassica napus* Jannin et al. (2012) characterized changes in gene expression after three days of treatment with humic acids. With microarray analysis, more than 300 genes from root and shoot tissues were identified to be differentially expressed in treated plants. According to DFCI annotations (http://compbio.dfci.harvard.edu) these genes were classified in nine clusters which covered the major metabolic functions in plants: general cell metabolism, nitrogen and sulphur metabolism, carbon metabolism and photosynthesis, stress responses, fatty acids, phytohormones, plant development, senescence and transport of ions and water. Carletti et al. (2008) carried out the first proteomics study on the effects of HS on plasma membrane (PM)-enriched root extracts. With 2D-PAGE analysis 63 spots were found to be affected by HS; in particular, 22 spots were upregulated and 38 were downregulated. Then, by liquid chromatography-tandem mass spectrometry (LC-MS/MS) 42 differentially expressed proteins were identified. Using the FunCat functional annotation scheme (Ruepp et al. 2004), the identified proteins were categorized in: energy and metabolism, cellular transport, transport facilitation and transport routes, interaction with environment, signalling, defence and cell rescue. Another proteomics study, similar to that one of Carletti et al. (2008), was conducted by Gao et al. (2015) to analyse the effects of water soluble humic material (WSHM) on the growth of *Bradyrhizobium liaoningense* $CCBAU_{05525}$ and its nodulation on soybean. From the 2D-PAGE analysis of *Bradyrhizobium liaoningense* cells, 15 up-regulated and 15 down-

regulated proteins were identified after treatment with WSHM. The differentially expressed proteins were then identified using a matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS), and it was reported they were involved in nitrogen and carbon metabolism, nucleic acid metabolism, signalling, energy production, and transmembrane transports. Metabolomics studies or studies targeting the effects of HS on subsets of plant metabolism are still scarce. Marino et al. (2013) observed metabolomics changes induced by low molecular weight humic acids (HA) on pear and quince leaf explants grown *in vitro*. Callus fragments were collected and metabolic profiles were detected through high resolution-magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy. 18 metabolites were identified in both leaf explants, and in treated samples HA enhanced the production of asparagine.

1.5 Fungicides with biostimulant-like activity

Fungicides are chemical and biological compounds used to kill pathogenic fungi or inhibit fungal spores germination (McGrath 2004) and represent the most used molecules, together with insecticides, in the seed coating practice of many crops.

Recently, more attention is given to direct influences of fungicides on the physiological processes of plants, besides the control on pathogens. It has been observed that several fungicides have also these additional "secondary effects" on plant physiology (Berdugo et al. 2012).

Wu and von Tiedemann (2001) studied the physiological effects of the strobilurin azoxystrobin (AZO) and the traizole epoxiconazole (EPO) on *Triticum aestivum* L. cv. Nandu. They highlighted that after fungicide application the senescence was significantly delayed due to an increase of the total superoxide dismutase (SOD, EC 1.15.1.1) activity and a reduction of superoxide (O_2^-) levels. Additionally, levels of hydrogen peroxide (H_2O_2) were significantly elevated in treated plants, playing a role as second messengers in inducing the expression antioxidant genes. Also in another study it was demonstrated that the application of BASF strobilurin F500 on wheat plants was able to enhance growth, nitrogen uptake and resistance to stress, and to delay senescence decreasing levels of Acetil-CoA carboxylase (ACC EC 6.4.1.2) enzyme and ethylene formation (Köhle et al. 2002). Fletcher et al. (2010) reported that azole fungicides have an influence on the physiology of treated plants, increasing the chlorophyll

content in winter wheat plants, delaying leaves senescence, and protecting plants from several abiotic stresses.

Some studies also demonstrated the influence of succinate dehydrogenase inhibitors (SDHIs) on plant physiology. Berdugo et al. (2012) reported that Bixafen (SDHI fungicide) application on wheat plants delayed the senescence of leaves, significantly enhanced the leaf green area duration, photosynthetic activity and grain yield. In treated plants, it was also observed a lower tissue temperature of leaves, which is a suitable indicator of tissue vitality and higher photosynthetic activity. The application of the SDHI fungicide isopurazam mixed with the triazole epoxiconazole was tested on wheat plants to study the effects on photosystem II (PSII) efficiency, biomass, and yield (Ajigboye et al. 2014). The mixture enhanced the efficiency of PSII photochemistry, associated to increment of CO_2 assimilation rate, stomatal conductance and transpiration rate. In treated plants, it was also observed a 28% increase in biomass and 4% increase in grain yield. These data were also confirmed by another the study of Ajigboye et al. (2017). Under drought conditions winter wheat plants treated with Sedaxane (novel SDHI seed treatment) had higher efficiency of PSII photochemistry, photosynthesis activity and biomass production compared to controls. From the analysis of the microarray study of sedaxane responsive genes emerged that most of the differentially expressed genes were involved in defence, chlorophyll synthesis and cell wall modification.

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2 Aim and Objectives

In the last decade, research and policymakers designed sustainable agriculture as the solution to the critical necessity to meet rising food needs and simultaneously to reduce environment impacts. One of the most innovative solutions to achieve these goals, is represented by biostimulant products, which at low doses efficiently increase plant yield and stress resistance.
Although already commercialized, these compounds are still unknown under many aspects. It is well documented that biostimulants induce several responses in treated plants and that these responses are a result of a complex network of action mechanisms. There is an urgent need among the scientific community and commercial enterprises to better elucidate the causal/functional mechanism of biostimulants.
This project aimed to study, by means of proteomics and metabolomics approaches, metabolic and biochemical status of plants treated with a known biostimulant, humic substances from earthworm *faeces,* highlighting proteins and metabolites involved in biological processes leading to increased plant growth. The elucidation of and action mechanisms of plant biostimulants will allow a development of second generation of biostimulants where synergies and complementary mechanism can be functionally designed.
Biostimulants can be obtained or extracted from several matrixes, such as protein hydrolisates, soil, compost, and mineral deposits products. Although humic substances with biostimulant effects from leonardites are really diffuse, studies on their impact on plant physiology and biochemistry are still scarce.
A second aim of the PhD project was to study and compare the biostimulant activity of humic substances extracted from different leonardites, analyzing their influence on root morphology and primary and secondary metabolism of treated plants. These results can provide new evidence and deepen the knowledge on these biostimulants that are considered “benchmark” in the humic substances biological activity research.
Finally, the third aim of this PhD project aimed to evaluate new compounds with possible secondary biostimulant effects. To this purpose, a succinate dehydrogenase inhibitor (SDHI) fungicide, generally seed-applied on maize, was tested in sterile condition to evidence possible plant growth promoting effects. The biostimulant activity of the fungicide could be an additional benefit, over and above its protective role against seed- and soil-borne diseases, which could be exploited in the cultivation of maize.

3 Manuscript I

Protein profiling of Arabidopsis roots treated with humic substances

Antonio Masi[1], Giovanni Battista Conselvan[1], Micaela Pivato[1], Giorgio Arrigoni[23], Tayyaba Yasmin[4], Sohaib Roomi [4], Paolo Carletti[1]*

[1] Dipartimento di Agronomia, Animali, Alimenti, Risorse Naturali e Ambiente (DAFNAE), Università degli Studi di Padova, Viale dell'Università 16, 35020 Legnaro (Padova), Italy

[2] Proteomics Center, Università degli Studi di Padova, 35131 Padova, Italy

[3] Department of Biomedical Sciences, Università degli Studi di Padova, 35131 Padova, Italy

[4] Department of Biosciences, COMSATS Institute of Information Technology, 44000, Islamabad, Pakistan

*corresponding author

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Abstract

Background and aim Humic substances (HS) influence the chemical and physical properties of the soil, and are also known to affect plant physiology and nutrient uptake. This study aimed to elucidate plant metabolic pathways and physiological processes influenced by HS activity.

Methods Arabidopsis roots were treated with HS for eight hours. Root proteins were studied by MS spectrometry coupled with iTRAQ (Isobaric Tag for Relative and Absolute Quantification) technique. 902 protein spots were identified for HS treated roots, where 291 proteins were differentially expressed. Bioinformatic tools such as DAVID, KEGG, IIS and Cytoscape were used to interpret the biological function, pathway analysis and visualization of the network amongst the identified proteins.

Results From this analysis it was possible to evaluate that most of the differentially expressed proteins were functionally classified into response to inorganic substances, redox homeostasis, energy metabolism, protein synthesis, cell trafficking and division.

Conclusions With the present study an overview of the metabolic pathways most modified by HS biological activity is provided. Moreover, from the analysis of interactomes and DAVID clusters it was possible to observe previously undiscovered HS effects, i.e. on the Ubiquitin and RACK1A interactome subnetworks.

Keywords

Proteomics, biostimulant, response to inorganic substances, ubiquitin, cell wall, redox homeostasis.

Introduction

Humic substances (HS) are complex, heterogeneous, mixtures of polydispersed organic compounds with different functional groups and molecular masses. These compounds represent the end products of microbial decomposition and chemical degradation of dead biota and are considered both the major components of soil organic matter (Nardi et al. 2002) and the most abundant naturally occurring organic molecules on Earth (Sutton and Sposito 2005). They are yet to be separated into pure components (Muscolo et al. 2013) while they are traditionally obtained from soils or sediments by means of dilute base solutions (Schnitzer and Monreal 2011).

HS influence the chemical and physical properties of the soil and its overall health as they participate in many agronomic, environmental, and geochemical processes (Nardi et al. 2009). HS are also known to affect plant physiology (Nardi et al. 2002) and the composition and function of rhizosphere microorganisms (Varanini and Pinton 2001). HS can be used directly on plants at low concentrations (Aguiar et al. 2012) given their well-known ability to enhance plant growth, yield and nutrient uptake. For this reason HS constitute a category of plant biostimulants as defined in du Jardin (2012) and Calvo et al. (2014).

The mechanisms of action of HS and other biostimulants are still debated. HS have been found to affect plant growth in different ways such as interacting with certain morphological and physiological processes related to plant growth (Jindo et al. 2011; O'Donnell 1973; Vaughan and Malcom 1985; Yang et al. 2004), root hair formation (Nardi et al. 2009; Schmidt et al. 2011), lateral root development and root hair formation (Nardi et al. 2009), and root cell elongation (Canellas and Olivares 2014). Some authors demonstrated that these positive effects could be due to an auxin-like effect (Canellas et al. 2011; Mora et al. 2014; Trevisan et al. 2010). Indole-3-acetic acid (IAA), a plant hormone of the auxin class, is known to have a pivotal role in root development, eliciting pericycle cells to re-enter the cell cycle and establish lateral root mitotic sites (Canellas and Olivares 2014). Moreover, the HS influence on IAA expression, coupled with NO-signalling (Zandonadi et al. 2010) increases plasma membrane (PM) H^+-ATPase activity in the root (Canellas et al. 2002; Nardi et al. 1991; Zandonadi et al. 2007), inducing cell-wall loosening, extension growth and nutrient uptake in root cells (Hager 2003). Other studies reported that HS also have an influence on root cell elongation and differentiation regulating the content of reactive oxygen species (ROS) (Garcia et al. 2016). HS are reported to upregulate glycolysis and tricarboxylic acid

cycle (TCA) (Nardi et al. 2007), nitrate uptake (Quaggiotti et al. 2004) and metabolism (Vaccaro et al. 2009) and phenylpropanoid metabolism (Schiavon et al. 2010).

In the last decade, new molecular "omics" approaches have been used to characterize the complexity of signal cascades and biochemical reactions responsible for the beneficial effects of HS on plant metabolism. Just a few papers (Carletti et al. 2008; Gao et al. 2015; Jannin et al. 2012; Trevisan et al. 2011) have been published using these techniques for analysing plant responses to HS. From the fundamental research perspective, studies on the transcriptomics and proteomics effects of HS will help to clarify how these biostimulants elicit plant growth, nutrient uptake, and stress-tolerance responses. Zandonadi et al. (2013) recently evidenced the need of efforts directed towards the development of standardized, accessible and cost-effective methods to quantify and qualify the biostimulant bioactivity. Such studies also would offer the potential to find biological markers to be used during product development (Calvo et al. 2014).

Despite the significant contribution of proteomic analysis in elucidating cell metabolism, proteomic analysis of HS treatment has been performed previously only in one study (Carletti et al. 2008) in maize root plasma membranes, based on 2D PAGE and gel image analysis. In the present work, we applied the iTRAQ (Isobaric Tag for Relative and Absolute Quantification) system to assess the changes in the proteome of Arabidopsis roots following HS treatment. With iTRAQ technique, peptides from previously digested protein samples are chemically labeled with isobaric tags, allowing the simultaneous identification and quantification of up to four or eight samples in the same MS/MS spectrum (Ross et al. 2004). Compared to classical proteomics based on 2D PAGE, iTRAQ labelling techniques present several advantages: i) a larger number of proteins can be identified; ii) relative quantification is more robust; iii) less abundant proteins are visualized, that cannot be seen in 2D gels; iv) study of hydrophobic proteins that cannot be easily separated in gel electrophoresis (Putz et al. 2012).

Within the more general aim of contributing to understand the physiological mechanisms underlying plant responses to HS, this work aims to highlight major biological functions or processes altered in response to HS biostimulation. To this scope proteomics data have been sifted by means of DAVID (Huang et al. 2009b), KEGG (Kanehisa et al. 2017; Kanehisa et al. 2016), IIS (Carazzolle et al. 2014) and Cytoscape (Cline et al. 2007) software.

Materials and Methods

Preparation of Humic Extract

The *faeces* of *Nicodrilus* [=*Allolobophora* (Eisen)=*Aporrectodea* (Oerley)] *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) (Minelli et al. 1995) were collected from the Ah horizon of an uncultivated couchgrass, *Agropyron repens* L., growing in soils classified as Calcaric Cambisol (CMc-F.A.O. classifi- cation) (FAO-UNESCO 1997). Earthworm culture conditions, HS extraction, and extract purification were conducted as reported in (Carletti et al. 2008). HS extraction was performed with 0.1M KOH (1:10 w/v) at 130 rpm for 16 h at 50 °C. The extracts were centrifuged at 7000 rpm for 30 min, and filtered on Whatman filter N. 2 paper (Whatman, Boston, USA). Humic extract was desalted by using 14 kDa cut-off dialysis Visking (Medicell, London, UK) tubing with distilled water. Distilled water was changed daily until neutral pH was reached (Conselvan 2017). Subsequently, the extracts were desalted on ion exchange Amberlite IR-120 (H^+ form), assessed for organic carbon content, and lyophilized before conducting the following analyses. Humic substances chemical characterization can be retrieved in Carletti et al. (2008).

Plants growth and treatment conditions

Arabidopsis (*A. thaliana*) plants were hydroponically grown in a growth chamber as described previously (Destro et al. 2011). After vernalization, Arabidopsis thaliana wild-type seeds were germinated in the dark before being transferred to the hydroponic system. Seedlings were grown in pools containing Murashige and Skoog basal salt mixure (Sigma-Aldrich) solution for 28 days, and the medium was changed every 7 days. Growth condition were: 14 hours of light at 20 °C, 10 hours of dark at 18°C and constant 60% relative humidity. After 28 days of pre-cultivation, plants were partitioned in two hydroponic system batches: one, containing Murashige and Skoog solution, was kept as control (CTRL), in the other 1 mg C/L of HS from earthworm *faeces* was added to the solution. After 8 hours of treatment, about 200 mg of plant roots (about 12 plants) were pooled and collected for each treatment, snap frozen in liquid nitrogen, and immediately treated for extraction. Two independent biological replicates were performed.

Root Protein Extraction

Protein extraction was performed as described by Lan et al. (2011). Briefly, roots (ca. 150-250 mg) were ground in liquid nitrogen; 50 mL pre-cooled acetone (-20°C), 10% TCA and

0.07% 2-mercaptoethanol were added and mixed vigorously. After 2 hours of precipitation at -20°C, proteins were collected by centrifuging at 35,000 *g* on an Avanti J-E with a JA-20 rotor (Beckman Coulter, Brea, USA) at 4°C for 30 min. The supernatants were removed, and the protein pellets were washed twice with cold acetone containing 0.07% 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride and a third time with cold acetone without 2-mercaptoethanol. Protein pellets were extracted using protein extraction buffer composed of 6 M urea, 50 mM triethylammonium bicarbonate, pH 8.5, and 2% CHAPS. In addition to the protocol of Lan et al. (2011), 1% PVPP was added to the extraction buffer to improve the purification from impurities such as polyphenols, and four cycles of sonication (10'' at 72 Hz, 10'' pause) were performed to improve protein solubilization. Protein extracts were then centrifuged at 19,000 *g* on an Avanti J-E with a JA-20 rotor (Beckman Coulter) for 20 min at 4°C. Eventually, supernatants were collected, and the protein concentrations were determined using a protein Bradford assay kit (Sigma Aldrich). Extracted proteins were then precipitated overnight with 80% acetone at -20 °C.

In situ Trypsin digestion and iTRAQ labeling

To further clean the sample from the detergent present in the extraction buffer, protein pellets were loaded into a pre-casted 4-12% SDS gel and the electrophoretic run was stopped as soon as the protein extracts entered the running-gel. They were then excised from the gels as single, narrow bands, and in-situ digestion and peptide extraction were performed as described elsewhere (Ahou et al. 2014). The resulting peptide solution was desalted on a C18 solid-phase extraction cartridge and 1 µg of each sample was analysed by LC-MS/MS to check the digestion efficiency (details of the instruments and instrumental methods are given in the following section). Peptides belonging to the two studied conditions (control and HS) were labelled with the iTRAQ reagents (ABSciex) according to the manufacturer's instructions, and for the two biological replicates swapping was used following the latin square experimental design. Prior to mixing the samples in a 1:1 ratio, 1 µg of each sample was analysed separately by LC-MS/MS (details of the instruments and instrumental methods are given in the following section). The resulting data were checked against the database setting the iTRAQ labelling as a variable modification. All the peptides were correctly identified as being iTRAQ-modified at the N-terminus and at each lysine residue. The samples were then pooled and dried under vacuum for following analysis.

Strong cation exchange fractionation

Strong cation exchange chromatography was performed on a strong cation exchange cartridge (AB Sciex) as previously described (Tolin et al. 2013). The labelled samples were dissolved in 500 µl of buffer A (10 mM KH_2PO_4, 25% acetonitrile, pH 3) and loaded onto the cartridge using a syringe pump with a 50 µL/min flow rate. The cartridge was washed 3 times with 500 µL of buffer A. Peptides were eluted in a stepwise manner with increasing concentrations of KCl in buffer A. The labelled peptides were eluted in eight fractions (500 µL per fraction) with the following concentrations of KCl in the buffer A: 50, 100, 120, 140, 160, 180, 200, and 350 mM. The volume of each fraction was reduced under vacuum to remove acetonitrile. Samples were desalted using C18 cartridges (Sep-Pack, C18, Waters, Milford, USA) according to the manufacturer's instructions. Samples were finally dried under vacuum and kept at -20 °C until MS analysis.

LC-MS/MS Analysis

Samples were re-suspended in H_2O/0.1% formic acid and 1 µg of each fraction underwent LC-MS/MS analysis. The MS analyses were conducted with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Pittsburgh, CA, USA) coupled online with a nano-HPLC Ultimate 3000 (Dionex- Thermo Fisher Scientific). Samples were loaded onto a homemade 10 cm chromatographic column packed into a pico-frit (75 µm id, 10 µm tip, New Objectives) with C18 material (ReproSil, 300 A°, 3 µm). Peptides were eluted with a linear gradient of acetonitrile/0.1% formic acid from 3% to 50% in 90 min at a flow rate of 250 nL/min. According to the method described by Kocher et al. (2009), the instrument performed a full scan at high resolution (60,000) on the Orbitrap, followed by MS/MS scans on the three most intense ions with CID fragmentation on the linear trap. MS/MS scans were performed on the same ions with higher energy collision dissociation fragmentation on the Orbitrap (with a resolution of 7,500) to obtain low mass range data suitable for protein quantification. The peptides reliably identified in each sample were inserted in an exclusion list that was used to perform (under the same chromatographic and instrumental conditions) a second LC-MS/MS run for each sample fraction. Nano-HPLC-MS/MS analysis was performed on a nanoAcquity system (Waters) connected to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Electron) equipped with a PicoView nanospray interface (New Objective). Peptide mixtures were loaded onto a 75-mm i.d., 25-cm length C18 BEH column (Waters) packed with 1.7-mm particles with a pore size of 130 A ° and were separated using

a segmented gradient in 90 min from 5% to 40% solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 300 nL min and a column temperature of 35_C. Solvent A was 0.1% formic acid in water. The LTQ-Orbitrap XL hybrid mass spectrometer was operated in positive ionization mode. The MS survey scan for all experiments was performed in the Fourier transform cell recording a window between 350 and 1,600 mass-to charge ratio (m/z). The resolution was set to 60,000 at m/z 400, and the automatic gain control was set to 500,000 ions. The m/z values triggering MS/MS were put on an exclusion list for 90 s. The minimum MS signal for triggering MS/MS was set to 5,000. In all cases, one microscan was recorded. For high-energy collision dissociation, the applied acquisition method consisted of a survey scan to detect the peptide ions followed by a maximum of three MS/MS experiments of the three most intense signals exceeding a minimum signal of 5,000 in survey scans. For MS/MS, we used a resolution of 7,500, an isolation window of 2 m/z, and a target value of 100,000 ions, with maximum accumulation times of 400 ms. Fragmentation was performed with normalized collision energy of 50% and an activation time of 30 ms.

Database Search and Quantification

The raw LC-MS/MS files were analysed using Proteome Discoverer 2.4 (Thermo Fisher Scientific). The software was connected to a Mascot Search Engine server, version 2.2.4 (Matrix Science, London, UK). The spectra were searched against an *A. thaliana* database (downloaded from ARATH Uniprot database version dated January 2013). Enzyme specificity was set to trypsin with two missed cleavages. Peptide and fragment tolerance was set to 10 ppm and 0.6 Da, respectively. Methylthiocysteine, 4-plex iTRAQ at the *N*-terminus and Lys were set as fixed modifications, while methionine oxidation was selected as a variable modification. Based on the search against the corresponding randomized database, false discovery rates (FDR) of 5% and 1% were calculated by the Proteome Discoverer. The data were pre-filtered to exclude MS/MS spectra containing less than 5 peaks or with a total ion count below 50. Were considered as positive hits all proteins identified and quantified with at least two independent peptides with a high degree of confidence (FDR 1%). The quantification was performed normalizing the results on the median value of all measured iTRAQ reporter ratios. The list of quantified proteins was exported to Excel for further filtering and statistical analyses, which were conducted after removing from the final list the proteins that showed a discordant trend in the replicates. A ratio of treated to control ≥ 1.3 (fold change $\geq +30\%$) was set as the threshold for increased content, while a ratio of treated to control ≤ 0.769 (fold change $\geq -30\%$) was taken to indicate decreased protein content.

Bioinformatics Analysis

Functional analysis of proteins was carried out by using DAVID V 6.7 (https://david.ncifcrf.gov; Huang et al. (2009a; 2009b)), a web based tool for functional annotation against large list of genes in order to understand the biological meaning behind the long list of genes. DAVID analysis was performed separately for up-and down-regulated genes. We used a list of genes that were found to be expressed in roots (Birnbaum et al. 2003) as background list in the study. The gene ontologies (biological process, molecular function and cellular compartment), protein domains and pathways were analysed. For the false positives linked with large list of genes, the output was filtered from DAVID on corrected *p*-values, using the Benjamini correction method ($p < 0.05$). The groups with highest enrichment scores have more biological significance than the groups with low enrichment score in the study. Only the clusters having an enrichment score ≥ 1.3 for both up and down-regulated genes were considered.

Identified proteins were analysed by means of KEGG Mapper – Search&Color Pathway on-line tool (http://www.genome.jp/kegg/tool/map_pathway2.html) (Kanehisa et al. 2017; Kanehisa et al. 2016) against Arabidopsis thaliana database using Uniprot ID as object.

Protein interactomes were built with Integrate Interactome System (IIS) platform (http://bioinfo03.ibi.unicamp.br/lnbio/IIS2/index.php; Carazzolle et al. (2014). Different interactome networks were built either based on the full list of identified proteins (Fig. S1; supplementary material) or based only on differentially expressed proteins (Fig. S2; supplementary material). In both cases networks were obtained either including only interactions between the input nodes or expanding the network to first neighbours' nodes (Bernardo et al. 2017). IIS output networks were visualized and analysed using Cytoscape 3.5.1 software (Shannon et al. 2003).

Results

iTRAQ labelling and Mass spectrometry

Following iTRAQ labelling, mass spectrometry analysis of HS treated samples of Arabidopsis roots resulted in identification of 902 different protein species, which were consistently found in the two biological replicates. Proteins with a fold-change ≥1.3 were considered as up-regulated and proteins with a fold-change ≤0.77 were considered as down-regulated proteins. In total 182 identified proteins were found to be over expressed, the ubiquitin-activating enzyme E1 1 (AT2G30110) being the highest with 3.1 fold change. Similarly, 110 proteins resulted to be repressed. The over and under expressed proteins are shown in Table S1 and Table S2, supplementary material, respectively.

The differentially expressed proteins were subjected to gene ontology *via DAVID platform* analysis in order to identify major biological processes involved in the response to HS. Pathway analysis and protein interaction network analysis were carried out to highlight the most regulated pathways and the interaction amongst the identified proteins. KEGG Pathway Most informative maps are TCA cycle (KEGG pathway entry: ath00020) (Fig. S3; supplementary material), Glyoxylate and dicarboxylate metabolism (ath00630) (Fig. S4; supplementary material), and Proteasome (ath03050) (Fig. S5; supplementary material).

Functional Classification, pathway and interactome analysis of differentially expressed proteins

DAVID functional annotation cluster analysis produced 16 enriched functional clusters for 182 up-regulated genes under high stringency conditions (Table 1). Response to inorganic substances is the most enriched cluster having enrichment score (ES) of 15.49. Other major groups of up-regulated proteins relate to protein synthesis / translation ES (8.87), metabolic process/respiration (ES 5.22), response to heat (E.S 3.85), cell wall macromolecule metabolic process (ES 2.12), cellular redox homeostasis (ES 1.99) and cell division (ES 1.36). Similarly, seven clusters were found for down regulated proteins (Table 2): response to inorganic substances (ES 4.75), nucleosome organization (ES 2.72), protein catabolic processes (ES 1.83) and peroxydase activity (ES 1.68).

Analyzing the 903 identified proteins with IIS platform and visualizing them with Cytoscape software, two different interactomes were obtained. One, including only interactions between the input nodes, consisted of 246 proteins (Fig. S2, supplementary material). The other one, where the network was expanded to the first neighbour nodes, was built with 1941 proteins

(Fig. S1, supplementary material). From the interactome of 246 proteins, it was possible to subdivide proteins for Cellular Component GO term (Fig. 1).

Table 1 List of the most significant clusters, with an enrichment score (ES) ≥ 1.3, for the up-regulated proteins. Cluster were produced using DAVID database.

Category	**GO ID**	**Name**	**ES**	**Proteins**	***p* value**	**Annotation cluster**
GOTERM_BP_FAT	GO:0010035	Response to inorganic substance	15.49	30	1.37E-13	1
GOTERM_BP_FAT	GO:0006412	Protein synthesis/ translation	8.87	25	1.54E-08	2
SP_PIR_KEYWORDS	-	Cell membrane	5.44	13	4.67E-05	3
GOTERM_BP_FAT	GO:0045333	Metabolic process/cellular respiration	5.22	9	7.54E-08	4
GOTERM_BP_FAT	GO:0006732	Coenzyme metabolic process	4.91	11	3.35E-05	5
GOTERM_BP_FAT	GO:0009408	Response to heat	3.85	6	0.0110	6
GOTERM_CC_FAT	GO:0043233	Organelle lumen	3.34	18	0.0011	7
KEGG_PATHWAY	ath00630	Glyoxylate and dicarboxylate metabolism	2.46	4	0.0323	8
INTERPRO	IPR018120	Glycoside hydrolase, family 1, active site	2.19	4	0.0013	9
INTERPRO	IPR017937	Thioredoxin, conserved site	2.18	4	0.0036	10
GOTERM_BP_FAT	GO:0044036	Cell wall macromolecule metabolic process	2.12	5	0.0018	11
GOTERM_BP_FAT	GO:0045454	Cell redox homeostasis	1.99	6	0.0147	12
SMART	SM00517	PolyA	1.95	3	9.88E-04	13
KEGG_PATHWAY	ath01066	Biosynthesis of alkaloids derived from terpenoid and polyketide	1.62	9	0.0305	14
GOTERM_BP_FAT	GO:0019725	Cellular homeostasis	1.52	7	0.0284	15
SP_PIR_KEYWORDS	-	Cell division	1.36	4	0.0373	16

Table 2 List of the most significant clusters, with an enrichment score (ES) ≥ 1.3, for the down-regulated proteins. Cluster were produced using DAVID database.

Category	GO ID	Name	ES	Proteins n.°	P. value	Annotation cluster
GOTERM_BP_FAT	GO:0010035	Response to inorganic substance	4.75	14	1.63E-05	1
SMART	SM00414	Histidone H2A	2.72	3	7.38E-04	2
GOTERM_BP_FAT	GO:0034728	Nucleosome organization	2.39	5	1.21E-04	3
GOTERM_BP_FAT	GO:0030163	Protein catabolic process	1.83	5	7.30E-04	4
SP_PIR_KEYWORDS		Peroxidase	1.68	4	0.0133	5
GOTERM_CC_FAT	GO:0005730	Nucleolus	1.47	8	0.0050	6
GOTERM_MF_FAT	GO:0045735	Nutrient reservoir activity	1.45	4	0.0094	7

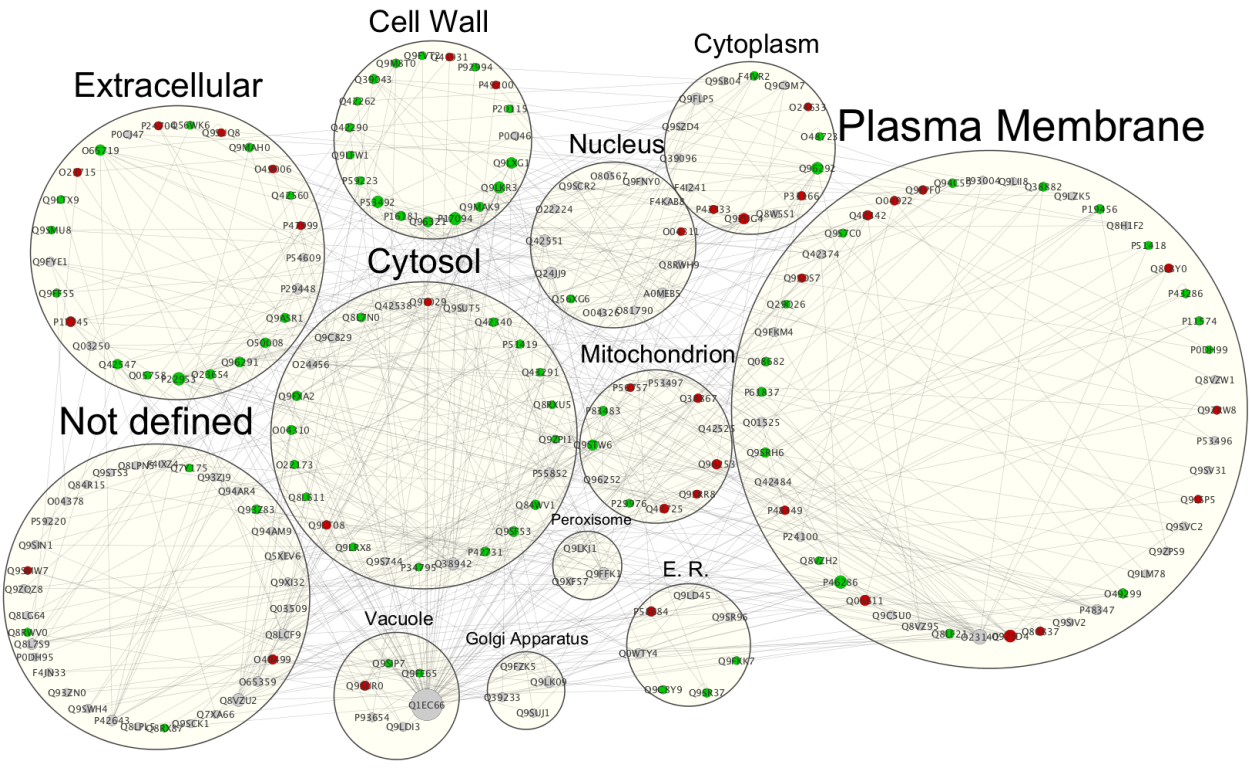

Fig. 1 Interactome of 246 proteins detected in Arabidopsis roots treated with HS. The interactome created with IIS website included only proteins with interactions between the input protein dataset. Proteins were visualized with Cytoscape 3.5.1 and they were distributed according to Cellular Components (GO) classification. Nodes marked in green were associated with significantly up-regulated proteins, red nodes denoted down-regulated proteins and grey nodes were proteins with no significant change. Labels report protein UniProt ID.

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Discussion

In order to decipher the metabolic readjustments that follow to the HS treatment, altered proteins are discussed in functional categories based on DAVID clusterization.

Response to inorganic substances

In this study, we identified a large number of proteins clustered as responses to inorganic substances and responses to Cd that were differentially expressed following HS treatment. Many of these are also involved in redox homeostasis, such as ROS scavenging as catalase 3 (AT1G20620), peroxidase 34 (AT3G49120) which were up-regulated and down expressed proteins as Glutathione S-transferase 1 (AT1G02930), Peroxidase 45 (AT4G30170), Peroxidase 49 (AT4G36430) and [Cu-Zn] Superoxide dismutase (AT1G08830) (details below in redox homeostasis paragraph).

The group also contains some of the overexpressed enzymes of glycolysis and TCA cycle as Aconitate hydratase 2 (AT2G05710), Aconitate hydratase 3 (AT4G26970) citrate synthase (AT2G44350) and Glucose-6-phosphate isomerase (AT5G42740) (see energy metabolism section). Similarly, some other identified proteins of diverse function were over expressed as heat shock cognate 70 kDa protein 1 (AT5G02500), heat shock cognate 70 kDa protein 3 (AT3G09440), protein disulfide-isomerase 2 (AT1G77510) and down regulated as proteasome subunit alpha type-3 (AT2G27020) and proteasome subunit alpha type-5-A (AT1G53850).

The most upregulated protein in our analysis was the ubiquitin-activating enzyme E1 (AT2G30110), involved in the first step in conjugating multiple ubiquitins to proteins targeted for degradation. Mutant analysis suggests that this gene is involved in defense response, and that ubiquitination plays a role in plant defence signalling (Bachmair et al. 2001; Hatfield et al. 1997)

Ubiquination importance in HS responsive proteins is also evidenced in IIS interactome (Fig. 2). Ubuiquitin UBQ3 is resulted to have direct interaction with 86 among differentially expressed proteins (57 up-regulated, 29 down regulated) for a total of 262 of the identified proteins.

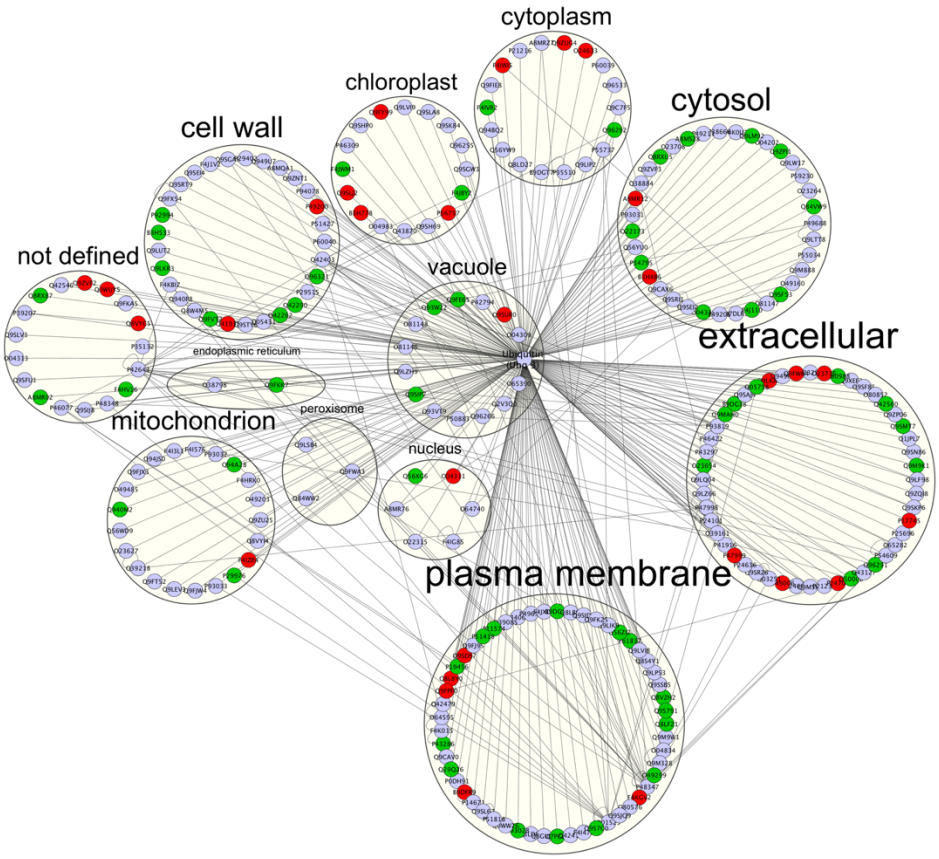

Fig. 2 Interactome of 262 proteins detected in Arabidopsis roots treated with HS directly interacting with Ubiquitin. The interactome created with IIS website included only proteins with interactions between themselves. Proteins were visualized with Cytoscape 3.5.1 and they were distributed according to Cellular Components (GO) classification, with Ubiquitin (Ubq3) as central node. Nodes marked in green were associated with significantly up-regulated proteins, red nodes denoted down-regulated proteins while grey nodes were proteins with no significant change. Labels report protein UniProt ID

The DAVID clusterization of our differentially expressed proteins as response to Cd is clearly incidental as no Cd was present in the nutrient solution or the HS, as confirmed by previous elemental analyses by ICP (data not shown). However, in soil, the functional moieties of HS such as alcoholic, carboxylic and phenolic groups play a vital role in homeostasis of metal ions by reversibly fixing the metals via S-containing ligands (Kalis et al. 2006). The increase in uptake of heavy metals with addition of HS was reported in plants in various studies (Evangelou et al. 2004; Halim et al. 2003), and is likely due to the presence of a high number of oxygenated functional groups in HS enabling interaction with metal ions, leading to the formation of complexes and that might interact with plant nutrition (Berbara and García 2014; Schiavon et al. 2010). This DAVID cluster is based on plant responses to

Cd pollution and HS-responsive proteins happen to belong to pathways highly represented in the plant responses to inorganic stimuli.

Redox homeostasis

We identified a number of proteins related to redox homeostasis whose expression increased in response to HS, including protein catalase 3 (AT1G20620), peroxidase 34 (AT3G49120), disulfide-isomerase 2 (AT1G77510), 2-Cys peroxiredoxin BAS1 (AT3G11630), nucleoredoxin-1 (AT1G60420), disulfide isomerase-like (PDIL) protein (AT3G54960) (Table S1; supplementary material). The proteins glutathione peroxidase 2, GPX2 (AT2G31570), Protein DJ-1 homolog A, DJ1A (AT3G14990), glutathione S-transferase U19 (GSTU19) (AT1G78380), glutathione S-transferase F7 (AT1G02920), Superoxide dismutase [Cu-Zn] 1, CSD1 (AT1G08830) resulted down-regulated by HS treatment (Table S2; supplementary material).

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), superoxide (O_2^-) and singlet oxygen ($^1O_2$) have been considered as an unavoidable process of normal aerobic metabolism in plants (Dinakar et al. 2010). Different cellular molecules including proteins, DNA, RNA and lipids may be destroyed by ROS (Shah et al. 2001), and plant cells have evolved enzymatic and non-enzymatic mechanisms against these deleterious effects of ROS (Zhang et al. 2009). Antioxidant enzymes such as CAT (EC 1.11.1.6), CSD (EC 1.15.1.1) and POD (EC 1.11.1.7) as well as other small molecules antioxidant (glutathione, ascorbic acid and carotenoids) are used against oxidative stress by plants (Tewari et al. 2008).

Our results confirm the study of García et al. (2012) which showed that the antioxidant system of rice roots responds to HA in a similar way as they do against stress. For example, the activity of CAT and POX were increased after 8 hours of treatment with HA. Muscolo et al. (1993) also reported the morphogenetic influence of HS on leaf explant of *Nicotiana plumbaginifolia* due to stimulation of peroxidases and esterase. The study of Cordeiro et al. (2011) also presents the up-regulation of ROS and CAT in maize after the application of HA extracted from Oxisol. However, when applied in combination with environmental stresses, such as drought (de Vasconcelos et al. 2009) or salinity (Aydin et al. 2012), also opposite effects on these proteins have been observed. Among the identified proteins 25 peroxidase isoforms were found (Table S1 and Table S2; supplementary material) of which PER34 (AT3G49120) was up-regulated while PER45 (AT4G30170) and PER49 (AT4G36430), were down-regulated. These proteins belong to Class III peroxidases, comprising catalytically

flexible enzymes with a great number of isoforms (a total of 73 in Arabidopsis), which have been found to regulate a wide range of physiological processes in plants such as cell wall metabolism, auxin catabolism, wound healing, generation of ROS. These enzymes use various electron donors such as phenolic compounds, auxin, secondary metabolites or lignin precursors for reduction of H_2O_2 (Passardi et al. 2005).

The contrasting expression level of peroxidase isoforms found in our results could reflect post translational modifications or different cellular localization of the isoforms. While it is known that HS stimulate root growth and this is accompanied by enhanced PRX activity, in this study we could detect a clear increase only in PER34; it seems noteworthy, to this regard, that Arabidopsis seedlings overexpressing *AtPrX34* exhibited significantly longer roots indicating its involvement in the reactions that promote cell elongation (Passardi et al. 2006). Taken together, these evidences point to PER34 as a major player in the peroxidase-mediated response to HS.

Protein disulfide isomerase 2 (PDI) (AT3G54960) was up-regulated in our study; PDIs are enzymes of the oxidoreductase family and are involved in the formation, rearrangement and reduction of disulfide bonds in proteins of eukaryotes (Cho et al. 2011). The proper folding of target proteins by PDI is necessary for the stability, trafficking, catalytic activity and communication with other proteins; PDI has been seen to be involved in many different physiological processes and responses to various types of stress (Lu and Christopher 2008).

Treatment with HS also resulted in lower expression of peroxiredoxin-2D (AT1G60740) while 2-Cys peroxiredoxin (2-Cys Prx) (AT3G11630), was up-regulated. Peroxiredoxins are ubiquitous thioredoxin-dependent peroxidases having cysteine as active site for reduction of peroxides, believed to play a chaperone function resulting from a redox-dependent conformational switch (Cerveau et al. 2016). Multiple sites of subcellular localization have been reported for peroxiredoxins suggesting a site-specific functions in plant cells (Dietz 2011), thus justifying the simultaneous up and down regulation of the two isoforms.

We identified three isoforms of dihydrolipoyl dehydrogenases (LPD), belonging to the group of redox homeostasis proteins, that are components of both mithocondrial and plastidic pyruvate dehydrogenase complexes (mtPDC and plPDC; Nishida 2007) converting pyruvate to acetyl-CoA and NADH. Only plastidial lipoamide dehydrogenase LPD1 (AT3G16950) was up-regulated, whereas the mitochondrial lipoamide dehydrogenase-1 (MTLPD1, AT1G48030), and the mitochondrial isoform dihydrolipoyl dehydrogenases-2 (MTLPD2, AT3G17240) were found unchanged as well as their partners in the PDC complex such as

pyruvate dehydrogenase (PDH; E1; AT1G24180) and dihydrolipoyl acetyltransferase (E2; AT3G13930).

Plant glutathione peroxidases (GPX) are ubiquitous in nature; in Arabidopsis eight GPX genes are found to be expressed in different plant compartments, tissues and developmental phases, and multiple signals can induce their expression. In this study, probable glutathione peroxidase-2 (AT2G31570) was down-regulated by HS. This enzyme has been reported to play a role in redox signalling transduction pathways (Passaia and Margis-Pinheiro 2015).

Proteins belonging to the redox homeostasis category have seemingly opposite expression patterns; some of them being up- and other down-regulated. One major reason explaining this apparent discrepancy is related with these proteins' subcellular localization (e.g. cytosolic vs. extracellular) and/or specificity for different substrates acting as electron donors, as in the case of peroxidases.

At a deeper glance, however, it is possible to draw also some additional considerations. ROS are likely to result from accelerated energy processes in glycolysis and TCA cycle, which are necessary for sustaining the increased ATP demand for biosynthetic processes due to HS stimulation. Up-regulation of ROS scavenging enzymes could then be interpreted as the need to counteract dangerous radicals generated by enhanced respiration. Down-regulation of several ROS metabolizing proteins may result in higher peroxide level, which in turn may act as a signal activating cell metabolic processes (Noctor et al. 2015), and interfere with auxin metabolism and cell wall remodelling. These effects are all known to be implicated in the response to HS. Moreover, Garcia et al. (2016) reported that ROS could act as signals. The ROS production, after HS treatment, create a Ca^{2+} gradient in the apical root region, leading to secondary root growth.

Energy Metabolism/Respiration

A large number of differentially expressed enzymes/proteins identified from Arabidopsis roots were found to be involved in plant energy metabolism.

Many of these enzymes are related to carbohydrate metabolism including glycolysis, TCA and pentose phosphate pathway and were over expressed such as aconitase 1 (AT4G35830), aconitase 2 (AT2G05710), aconitase 3 (AT4G26970), glucose-6-phosphate 1-dehydrogenase (AT5G40760), citrate synthase 4 (AT2G44350), succinate dehydrogenase [ubiquinone] flavoprotein subunit 1 (AT5G66760), phosphoenolpyruvate carboxylase 1 (AT1G53310) and phosphoenolpyruvate carboxylase 3 (AT3G14940), while the enzyme malate dehydrogenase

(AT3G15020) was repressed in this study. These results are briefly described in KEGG Pathways (Fig. S3; supplementary material)

The glycolytic pathway is important in plants as it provides fuel for respiration and major carbon skeletons for the synthesis of various vital compounds such as nucleic acids, amino acids, fatty acids, isoprenoids and other secondary metabolites (Plaxton 1996). The role of glycolysis in opposing various stresses including drought, salt, cold and anoxia has been widely reported in literature (Kosova et al. 2014). Thus, the known HS effect on plant stress relief (Aguiar et al. 2016) can be, at least partially, ascribed to their action on the glycolytic pathway.

One of the up-regulated enzymes in our study is glucose-6-Phosphate dehydrogenase (G6PD) (AT5G40760), catalyzing the oxidation of glucose-6-phosphate to 6-phosphogluconate, the key step in the pentose phosphate pathway (PPP). PPP is the primary source of NADPH in various biosynthetic processes such as fatty acid metabolism, integration of nitrogen into amino acid and resistance against oxidative destruction. An intermediate in PPP, ribose-5-phosphate is used for phenylpropanoid production through shikimate pathway (Scharte et al. 2009). G6PD is highly regulated: besides transcriptional control, also redox regulation and cellular NADPH/$NADP^+$ ratio have been shown to regulate the activity of diverse G6PD isoforms (Schurmann and Buchanan 2008). In a previous study, the low molecular weight humic extracts were found to stimulate the Pi level and energetic metabolism, resulting specifically in higher Glucose-6-phosphate and ATP level (Zancani et al. 2009).

In our study two isoforms of Phosphoenolpyruvate carboxylase (PEPC-1) (AT1G53310) and PEPC-3 (AT3G14940) were up-regulated. PEPC (EC 4.1.1.31) is the key enzyme which stimulates the irreversible beta-carboxylation of PEP to produce oxaloacetate and inorganic phosphate (Pi) in the presence of HCO_3. PEPC is allosterically inhibited by L-malate and is activated by its positive effector, Glc-6-P. It is further regulated by both reversible phosphorylation by the enzyme PPCK (PEPC kinase) and by monoubiquitination (Shi et al. 2015). In plant, non-photosynthetic tissues PEPC ensures an anaplerotic flux of intermediates to TCA cycle. Consistently with our results, Muscolo et al. (2007) have also shown an enhancement of Phosphoenolpyruvate carboxylase activity in response to HS.

Malate dehydrogenase (MDH, EC 1.1.1.37) is an important regulatory enzyme of energy metabolism in eukaryotes and catalyzes the conversion of malate to oxaloacetate by using NAD as cofactor. Eukaryotic cells possess two isoforms of MDH, one at mitochondria and the other at cytosol. Mitochondrial MDH is used in TCA cycle while cytosolic MDH participates in gluconeogenesis or shuttle systems for transferring the reducing equivalents

across mitochondrial membrane (Goward and Nicholls 1994). In our study five isoforms of MDH were identified but only one mitochondrial isoform mitMDH-2 (AT3G15020) was down-regulated while the others were not changed. Similar results of down regulation of MDH in maize root cell membrane extracts after HS application was described by Carletti et al. (2008). In maize leaf, enzymatic extracts, Nardi et al. (2007) reported the enhancement in MDH activity after treatment with HS fractions. However, enzyme extract activity essay does not distinguish the MDH isoforms; furthermore, different regulation might occur in leaves compared to roots.

In mitochondria, aconitase (ACO; EC 4.2.1.3) plays an important role in TCA cycle by catalyzing the isomerization reaction of citrate to isocitrate via cis-aconitate. The cytosolic isoform is involved in glyoxylate cycle (Moeder et al. 2007). We identified three isoforms of aconitase which were all upregulated: aconitase 1 (AT4G35830, cytoplasmic and mitochondrial), aconitase 2 (AT2G05710, mitochondrial) and aconitase 3 (AT4G26970, mitochondrial). Adjacent spots of aconitase were also reported as differentially expressed by Carletti et al. (2008) in HS-treated maize roots. Our results are also supported by the study of Trevisan et al. (2011) in which gene expression of metabolic enzymes including aconitase was induced after treatment with HS.

Another enzyme that was up-regulated in our current study is citrate synthase 4 (AT2G44350). In all organisms, citrate synthase is a key enzymes in TCA cycle catalyzing the condensation reaction of oxaloacetate and acetyl-CoA to form the six-carbon citrate (Schmidtmann et al. 2014). Increase in citrate synthase activity by HS was observed in maize seedlings (Nardi et al. 2007).

Flavoprotein subunit 1 (AT5G66760), a component of the mitochondrial succinate dehydrogenase complex (EC 1.3.5.1), was also increased in our study. Succinate dehydrogenase is involved in both the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol, respectively (Figueroa et al. 2001).

ATP-citrate lyase (ACL) catalyzes the formation of acetyl-CoA and oxaloacetic acid from citrate and CoA in the cytosol. The ACL in plant is composed of two subunits, ACLA and ACLB. Here, we observed higher expression of ATP-citrate lyase subunit B (AT3G06650) after HS treatment. ACL is necessary for normal growth and development and is not compensated for by any other source of acetyl-CoA in cell. The decreased level of this enzymes resulted in reduction of cytosolic acetyl-CoA–derived metabolites (Fatland et al. 2005).

Taken together, the activation of expression of the above mentioned enzymes involved in glycolysis, pentose phosphate pathway and TCA cycle due to HS may result in an increase in the production of NAD(P)H, ATP and carbon skeletons needed for various vital cellular processes such as biosynthesis of macromolecules (proteins, nucleic acids, amino acids, fatty acids, secondary metabolites), which, in turn may explain the known effect of HS on plant growth.

The stimulation of energy metabolism related enzymes after HS treatment has been reported previously by many researchers: up-regulation of various metabolic processes and signalling pathways associated to plant development (Pizzeghello et al. 2013; Trevisan et al. 2010), increase in photosynthesis and respiration in plants (Jannin et al. 2012; Nardi et al. 2002), stimulation of glycolysis and TCA cycle related enzymes in maize (Nardi et al. 2007) and in *Pinus nigra* callus (Muscolo et al. 2007). Quaggiotti et al. (2004) also found that HS can stimulate carbon and nitrogen metabolism by overexpression of various enzymes of glycolysis and TCA cycle. In another study, the effect of HS on phosphate level and energy metabolism was described in tobacco suspension culture in which the HS, small in size and hydrophilic in nature, were found to stimulate the Pi level and energetic metabolism, resulting specifically in higher Glucose-6-phosphate and ATP level (Zancani et al. 2009).

Cell wall metabolism

Cell wall is contained in the outermost extracellular matrix in plant cells and its regulation is important for proper size and shape, mechanical resistance, interaction with environment, defence against pathogens, development and growth (Reiter 2002). Cell wall is the first compartment getting in contact with the exogenous agents, thus unsurprisingly its proteome resulted modified by the HS-treatment. In our study, many of differentially expressed enzymes related to cell wall metabolism were up-regulated (Fig. 1), as for example the enzyme glycerophosphodiester phosphodiesterase (GDPD) (AT5G55480) (EC 3.1.4.46). This enzyme plays a vital role in many physiological processes in living organisms, by converting glycerophosphodiester to glycerol-3-phosphate and alcohols during glycerol metabolism (Cheng et al. 2011). GDPD and its homologs have been found to be involved in cell wall organization and in root hair morphogenesis in Arabidopsis (Hayashi et al. 2008). An important cell wall related protein which was also up-regulated is RAF1 (AT3G10740), bifunctional alpha-L-arabinofuranosidase/ beta-D-xylosidase. RAF1 is located in roots and shoots in many types of cells, in particular in vascular tissues (Chavez Montes et al. 2008). Alpha-xylosidase (AT1G68560) was increased after treatment with HS. This enzyme is

responsible for removing xylosyl residues from xyloglucan oligosaccharides and triggers xyloglucan mobilization. Xyloglucan is the central hemicellulose in primary cell walls of most seed plants and plays an important role in regulating and separating the cellulose microfibrils during growth (Sampedro et al. 2010). These evidences point to readjustments in cell wall composition which are likely required in remodeling and redefining the root organ size, architecture and root hair morphogenesis when stimulated by HS (as found in Trevisan et al. (2010)).

Protein synthesis, folding, and degradation

The involvement of HS in stimulation of protein synthesis in plants has been previously observed in many studies: in Arabidopsis roots (Trevisan et al. 2011), maize roots (Carletti et al. 2008) and guava leaves (Dantas et al. 2007). Canellas et al. (2002) also observed that the HA-IAA groups may be able to access receptors and resulted in activation of protein synthesis in maize roots.

Our differentially expressed proteins comprise 26 ribosomal proteins (RPs) mostly up regulated. Ribosomes are the basic and essential components of every cell and catalyse numerous transpeptidal esterase reactions during protein synthesis. Ribosomal proteins (RPs) are not only vital for protein synthesis but also play a central role in cell division, growth and metabolism. The role of RPs as regulatory components in addition to their housekeeping function in developmental processes has been indicated by various mutational studies (Byrne 2009).

Ribosomal proteins are regulated by various regulators: for example, the application of BAP (cytokinin) and IAA (auxin) increased the transcription of RPS15aF while ABA treatment decreased it. Abiotic stresses like temperature and mechanical stress increased the transcript of RPS15aA, RPS15aD and RPS15aF (Hulm et al. 2005).

This could suggest an overall increase in ribosome production in HS treated root, which is consistent with enhanced protein synthesis leading to higher biomass production.

From the analysis of the interaction between the differentially expressed proteins of the interactome, we highlighted an interesting network, which has as central node RACK1A (AT1G18080) protein. RACK1A is a scaffold protein, formed by WD40 repeats, which can simultaneously interact with several molecules (Nilsson et al. 2004). It is located on the back of 40S ribosome subunit and, interacting with eIF6 (AT3G55620 or AT2G39820) protein, it could directly regulate protein translation (Adams et al. 2011; Guo et al. 2009). RACK1A gene expression is controlled by growth promoting extracellular stimuli, suggesting the role

of this protein in the cellular adaptation processes that occur during cell division (McCahill et al. 2002). In our study RACK1A was not significant differentially expressed, but it could be a central node for the influence of HS on cell responses. Based on the study of Guo et al. (2011) it can be hypothesized that HS influence the expression of proteins which respond to redox homeostasis: GPX2 (AT2G31570), DJ1A (AT3G14990), CSD1 (AT1G08830), GTU19(AT1G78380). Signals might be received by RACK1A which directly interacts with ribosomal and cytoskeleton proteins: RPL21A (AT1G09590), RPL25E (AT4G39200), RPL27C (AT4G15000), ACT2 (AT3G18780) (Fig. 3).

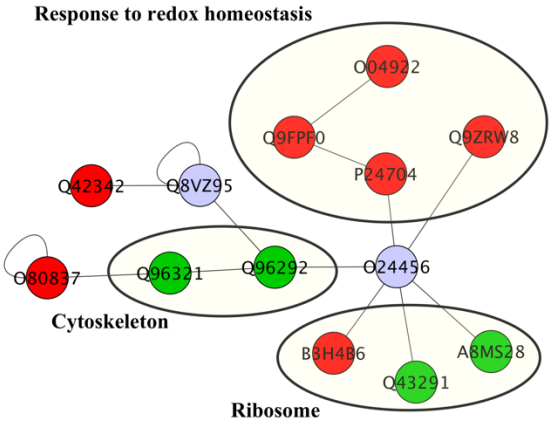

Fig. 3 Interactome sub-network with RAC1A protein (Q24456) as central node. this sub-network was obtained from the interactome of 246 proteins detected in Arabidopsis roots treated with HS. Nodes marked in green were associated with significantly up-regulated proteins, red nodes denoted down-regulated proteins and grey nodes were with no significant change. Labels report protein UniProt ID.

The enzyme lysyl-tRNA synthetase (AT3G11710), which is up-regulated in our experiment, has a key role in conversion of genetic information from mRNA to protein by catalyzing the formation of Lysyl-tRNA (Freist and Gauss 1995). The enzyme is also found to be linked with many other secondary functions such as activation of gene expression (Lee et al. 2004), and by serving as a cytokine (Park et al. 2005).

Our data report an increase of several isoforms of poly(A)-binding protein (PABP2, AT1G49760; AT2G23350; AT4G27000; AT4G34110). After attaching with 3' end of mRNA, PABP interacts with eukaryotic translation initiation factor 4F complex enhancing the translation process inside the cell (Sachs and Varani 2000). The role of PABP is also found to be correlated with nuclear export of mRNAs (Brune et al. 2005) and stability (Behm-Ansmant et al. 2007). In Arabidopsis thaliana, a total of eight different isoforms of PABP have been characterized (Belostotsky 2003).

Various heat shock protein cognates (AT4G24280, AT5G49910, AT1G79930, AT3G09440) were also over produced in response to HS. The 70-kD heat shock proteins (Hsp70s) are found in all cellular compartments of almost all organisms and have been found to be crucial for protein folding, protein translocation, and stress responses (Latijnhouwers et al. 2010). Two of the up-regulated Hsp70S have been described in Arabidopsis as stromal isoforms cpHsc70-1 (At4g24280) and cpHsc70-2 (At5g49910). In one knockout study a mutation in cpHsc70-1 resulted in abnormal leaves, impaired root development and retardation in growth (Su and Li 2008b).

Another up-regulated protein belonging to the class of protein folding is the Luminal-binding protein 1 (AT5G28540), also known as BIP1. Immunoglobulin-binding proteins (BiP) are molecular chaperones of the Hsp70 family and have a key role in regulation and control of translocation and folding of proteins inside the ER. Three genes as BIP1, BIP2 and BIP3 are found in Arabidopsis; BIP1 and BIP2 being ubiquitous in nature, while BIP3 has been shown to be expressed only under stress conditions in the majority of organs. Many stresses, both biotic and abiotic such as fungus, insect, cold, drought and nutrient deficiency were seen to induce BIP expression (Alvim et al. 2001; Chen and Lee 2011).

Plant proteasomes (26S proteasomes) contain two subparticles: the core particle (CP) or 20S proteasome where proteins are degraded, and the regulatory particle (RP) or 19S proteasome. 26S proteasomes are in charge of the ATP-dependent degradation of ubiquitin tagged proteins (Sadanandom et al. 2012) including normal, mutated, misfolded and damaged proteins (Kurepa and Smalle 2008; Tolin et al. 2013). The ubiquitin-proteasome system plays a role in nearly all aspects of cell homeostasis including plant development, response to plant hormones (Sullivan et al. 2003) and signalling in response to abiotic and biotic stimuli (Smalle and Vierstra 2004). Moreover, the 20S particle can also degrade proteins in a ubiquitin-independent manner, mainly for oxidized proteins (Foyer and Allen 2003). In this study HS treatment resulted in differential expression of some proteasome subunits such as 26S proteasome non-ATPase regulatory subunit 14 (AT5G23540), Proteasome subunits alpha type-3 (AT2G27020), alpha type-5-A (AT1G53850), beta type-2-B (AT4G14800) and beta type-7-A (AT3G27430) all of which are down-regulated and belong to the 20S (CP) subparticle, and the 26S protease regulatory subunit S10B homolog B (AT1G45000) which is up-regulated and belongs to the 19S (RP) subparticle. The involvement of the proteasome complex may be seen as a component of cell metabolic remodelling in the response to HS treatment, coherently with the view that the proteolytic capacity of a cell is the result of a

careful balancing act that reflects environmental conditions and developmental stage (Kurepa and Smalle 2008)

26S proteasome is ubiquitin and ATP dependent proteasome and is involved in protein degradation in the nucleus and the cytosol (Voges et al. 1999; Pickart and Cohen, 2004). Various catalytic activities such as trypsin-like, chymotrypsin-like peptidylglutamyl-peptide hydrolase were performed by 20S core particle of 26S proteasome (Voges et al. 1999; Groll et al. 1997). In Arabidopsis 26S proteasome are found to be involved in both basal defense and R gene mediated defense (Yao et al. 2012).

This observation is confirmed by KEGG pathways analysis, pointing to the majority of the identified ribosomal proteins being up-regulated while those of the proteasome (Fig. S5; supplementary material) being down-regulated.

All these observations indicate that up-regulation of ribosomal proteins is a major HS effect in Arabidopsis roots, which may be related with enhanced protein synthesis which is required to sustain growth.

Cell Trafficking and division

DAVID analysis on our proteomic data identified many differentially expressed proteins related to cell vesicle trafficking and growth such as Actin-7 (AT5G09810), Actin-2 (AT3G18780), Dynamin-related protein 1C (AT1G14830), Patellin-1 (AT1G72150), Patellin-2 (AT1G22530), Patellin-4 (AT1G30690), all of which were up-regulated. Two other proteins involved in actin metabolism were also found to be upregulated, namely GDPDL4 (AT5G55480), reported to be involved in actin nucleation and VHA-B1, V-type proton ATPase subunit B1 (AT1G76030), involved in actin filament assembly (Ma et al. 2012). The involvement of actin in plant responses to HS has been already highlighted in previous works (Carletti et al. 2008) as well as transport processes and vesicles trafficking related genes (Trevisan et al. 2011).

The role of actin in cellular processes is diverse and ranges from cell division and morphogenesis to cell motility (Pollard and Cooper 2009). Actin is also found to be important for tip growth (polarized cell extension) in plants (Menand et al. 2007), whose implications are of particular relevance in this study.

Dynamin-related protein 1C (AT1G14830) was also over expressed in this study. Dynamin is the key component of clathrin-mediated membrane trafficking, crucial for numerous stages of growth and development in plants. Unlike other organisms, plants require both classical

dynamin (DRP2) as well as dynamin-related proteins (DRP1) for clathrin-mediated membrane trafficking (Backues et al. 2010).

Patellin is a phosphoinositide-binding protein that plays a role in membrane trafficking during the expansion and maturation stages of cytokinesis, in particular cell-plate formation (McMichael and Bednarek 2013).

Among the proteins implicated in cell division processes also the HSP90-like protein GRP94 (also known as SHD, AT4G24190) might be considered. While its fold change of 1.23 suggests some up-regulation by HS, it is slightly lower than the selected threshold of >1.3 for consideration in our current study; however, it seems relevant to us that this gene was found to be highly expressed in (Trevisan et al. 2011). GRP94 is a glucose-regulated protein of endoplasmic reticulum and is involved in various developmental and physiological functions in multicellular organisms (Marzec et al. 2012); Arabidopsis plants with silent GRP94 showed abnormal phenotype in roots and shoot meristem (Ishiguro et al. 2002).

Heat response

HS caused a significant increase in expression of heat responsive proteins in this study: heat shock 70 kDa protein 6 (AT4G24280); Heat shock 70 kDa protein 3 (AT3G09440); Heat shock 70 kDa protein 7 (AT5G49910); Probable mediator of RNA polymerase II transcription subunit 37e (AT5G02500); Mediator of RNA polymerase II transcription subunit 37a (AT5G28540); Lipocalin (AT5G58070).

The 70-kD heat shock proteins (Hsp70s) are molecular chaperones involved in a variety of cellular processes including protein folding, protein transport across membranes, modulation of protein activity, regulation of protein degradation, and prevention of irreversible protein aggregation. Plant Hsp70s are encoded by a multiple-gene family (Su and Li 2008a). It is well-known that Hsps are ubiquitous proteins found in plant and animal cells, which were initially described to be involved in heat shock, but they are known to be induced by a wide variety of stresses, including cold, drought, salt, UV-light, wound, and biotic stresses (Wen et al. 2017).

Lipocalin (TIL, AT5G58070) is another heat responsive protein which was overexpressed in this study. Lipocalins are small extracellular proteins and have been implicated in freezing and oxidative stress tolerance in Arabidopsis (Charron et al. 2008), probably by contrasting lipid peroxidation triggered by heat stress as shown in Arabidopsis TIL1 knockout mutants (Chi et al. 2009).

Chromosome organization

The eukaryotic cells package and organize the nucleoprotein into a complex structure called chromatin. The basic unit of chromatin is called nucleosome and consists of the genomic DNAs wrapped over two copies of four histone molecules: H2A, H2B, H3, and H4 (Schneider and Grosschedl 2007). Biological studies showed that histone variants are involved in numerous processes including cell cycle, DNA damage response and transcription (Arnaudo et al. 2011). Cellular machinery can change specifically the histone molecules to regulate the gene expression (Heyse et al. 2009). The adjustment of histone molecules in order to monitor gene expression is very important for cell wall development (Dhawan et al. 2009), flowering time control (Cao et al. 2008), seed development (Liu et al. 2007), root growth (Yao et al. 2013) and plant defense responses (Palma et al. 2010; Xia et al. 2013). The modification of histone by methylation and ubiquitination can either activate or repress transcription depending upon their targets (Berke et al. 2012; Quan and Hartzog 2010).

In this study, treatment with humic substances resulted in repression of Histone H2B.11 (AT5G59910), Histone H2B.6 (AT3G45980), Probable histone H2A.7 (AT5G59870) and Probable histone H2A.2 (AT3G20670), while Histone H2A variant 1 (AT3G54560) is up-regulated. The rationale behind the existence of multiple genes for the same histone protein is unclear. The reasons for the presence of multiple histone genes may be either simply for backup in case of inactivating gene mutations or may be required for synthesis of multiple histones needed for chromatin assembly during DNA replication. The simultaneous reduction in expression of histones is probably related to transcriptional control resulting in coordinated cell cycle and root growth.

Integration with previous results and validation of proteomic analysis results

In this work we have provided a thorough proteomic analysis in Arabidopsis roots treated with HS obtained at our laboratory, following a standardized procedure for extraction, purification and characterization providing a product with homogeneous and stable properties over time.

Identical experimental conditions in terms of HS quality, concentration, exposure time, growth chamber settings (temperature, humidity, daylength) were previously used to treat Arabidopsis plants also in another study, aimed at studying the effects of HS on gene expression by a transcriptomic approach based on the detection of cDNA-AFLP markers

(Trevisan et al. 2011). When comparing our proteomic results with that transcriptomic analysis, we found an overall similar effect in terms of proportion of up-regulated to down-regulated genes/proteins: while 63% of total altered proteins were up-regulated, 75% of differentially expressed genes were up-regulated in the study from Trevisan et al. (2011). Together, these evidences point to a predominant effect of HS in up-regulating gene expression.

More in details, four enzymes that are up-regulated in our study (AT3G16460, AT4G35830, AT2G26080, and AT1G57720) were also found to be up-regulated at gene expression level.

Those results properly represent an independent confirmatory evidence and validation of our current experimental results by alternative gene expression analysis. It is expected that more confirmations would come from genome-wide microarray or RNA-seq data analysis, bearing in mind that due to post-transcriptional and post-translational modifications, and different protein species stability and turnover rate, it is not always possible to directly correlate transcript with protein expression levels.

Conclusions

With the present work, an overview of metabolic pathways influenced by HS activity is presented, only in part previously observed. Taken together, our results point to the activation of enzymes involved in glycolysis, pentose phosphate pathway and TCA cycle to support the production of NAD(P)H, ATP and carbon skeletons needed for various vital cellular processes. Stimulation of energy metabolism may explain the known beneficial effects of HS on plant growth. Up-regulation of ROS scavenging enzymes may be interpreted as the need to counteract dangerous radicals generated by accelerated energy processes in glycolysis and TCA cycle. Up-regulation of ribosomal proteins, HSPs and actin are likely representative of a co-ordinately enhanced protein synthesis, folding, trafficking and transport across membranes which is required to sustain growth. Our findings also point to readjustments in cell wall composition which are likely to be required in root remodelling and root hair morphogenesis. Finally, the interactome analysis highlighted the possible role of RACK1A as a central node in cell responses to HS.

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Supplementary material

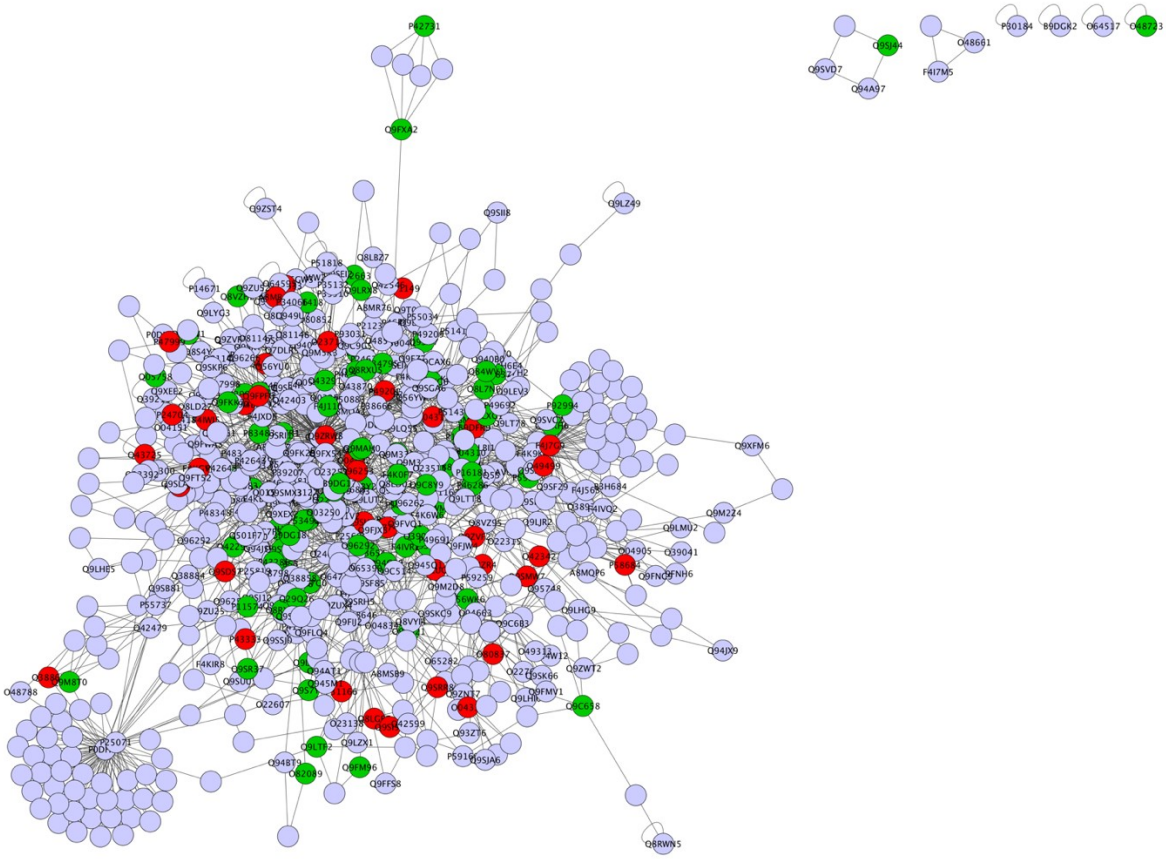

Fig. S1 Interactome of 903 detected proteins in Arabidopsis roots treated with HS. The network was built with ISS website, using first neighbors expansion, deletion of nodes with degree 0 and 1. Nodes marked in green were associated with significantly up-regulated proteins, red nodes denoted down-regulated proteins and grey nodes were proteins with no significant change. Labels report protein UniProt ID. Not labelled proteins are first neighbors (see Carazolle et al., 2014).

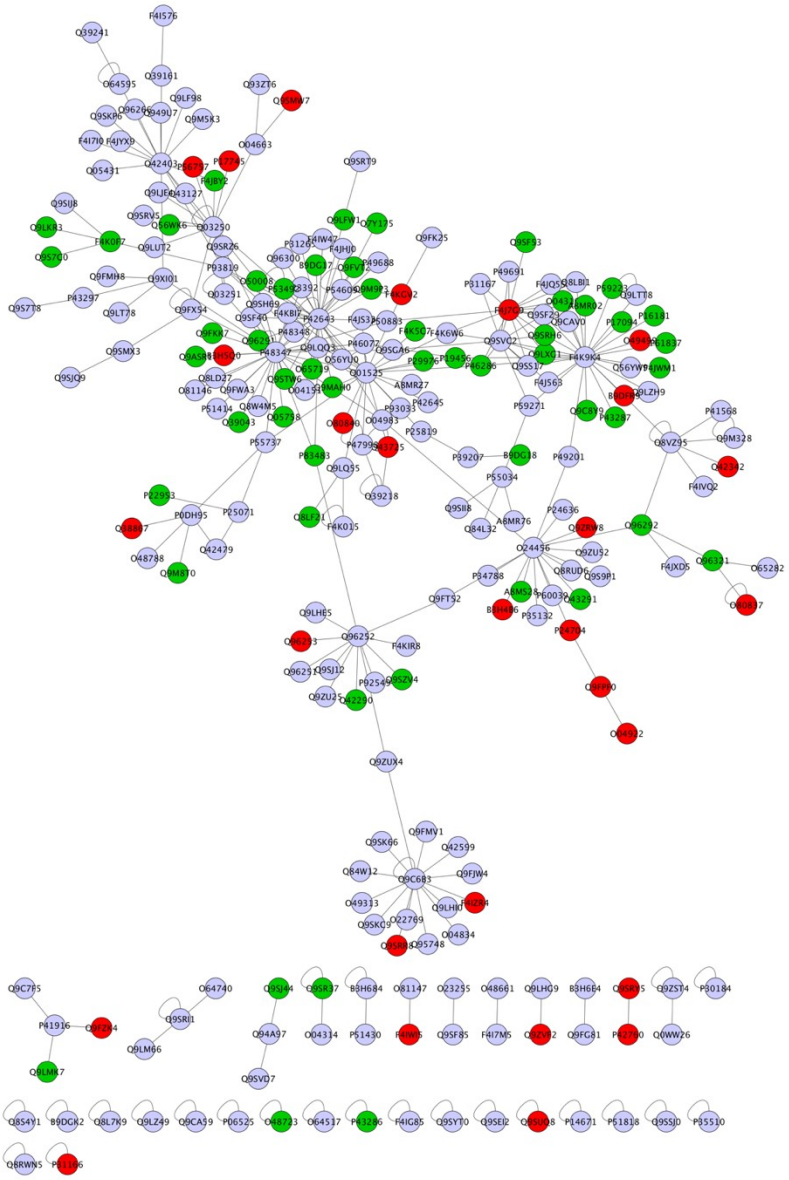

Fig. S2 Interactome of differentially expressed proteins detected in Arabidopsis roots treated with HS. The interactome created from IIS website included only proteins with interactions between themselves and first neighbors (Carazolle et al., 2014; Bernardo et al., 2017). Nodes marked in green were associated with significantly up-regulated proteins, red nodes denoted down-regulated proteins and grey nodes were proteins with no significant change. Labels report protein UniProt ID.

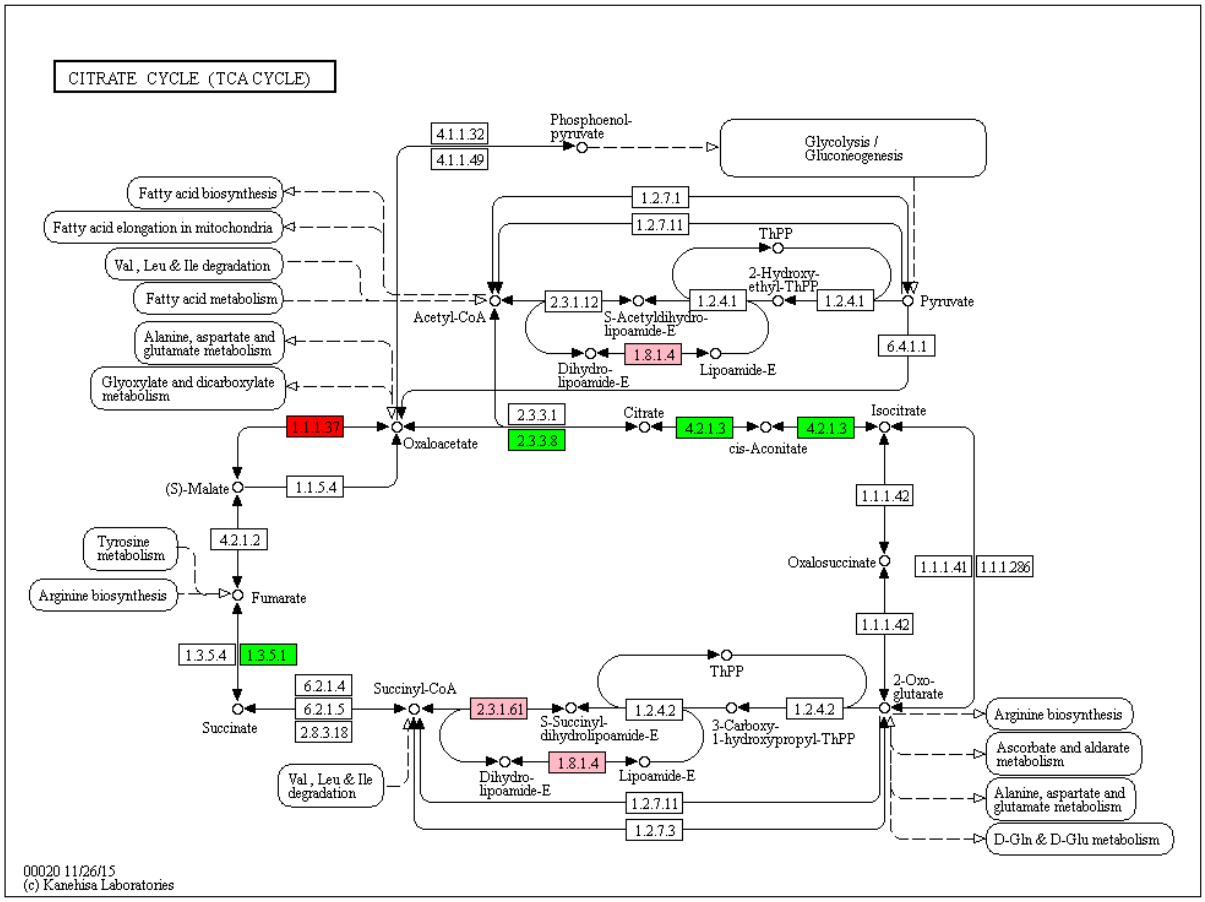

Fig. S3 Regulatory changes on the pathways of Citrate cycle (TCA cycle), with highlighted up-regulated proteins (green), down-regulated proteins (red) and proteins without fold-change (pink). Labels report EC numbers.

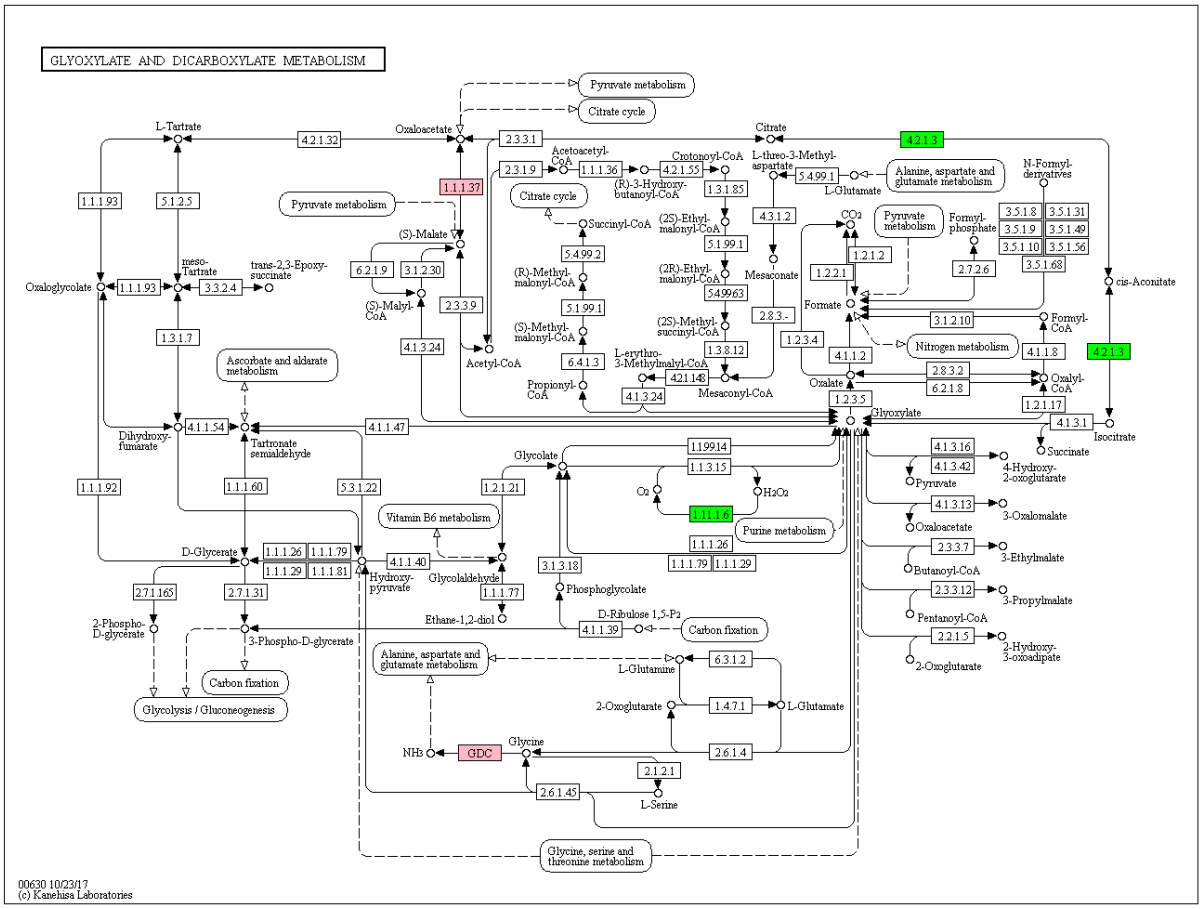

Fig. S4 Regulatory changes on the pathways of glyoxylate and dicarboxylate metabolism, with highlighted up-regulated proteins (green) and proteins without fold-change (pink). Labels report EC numbers.

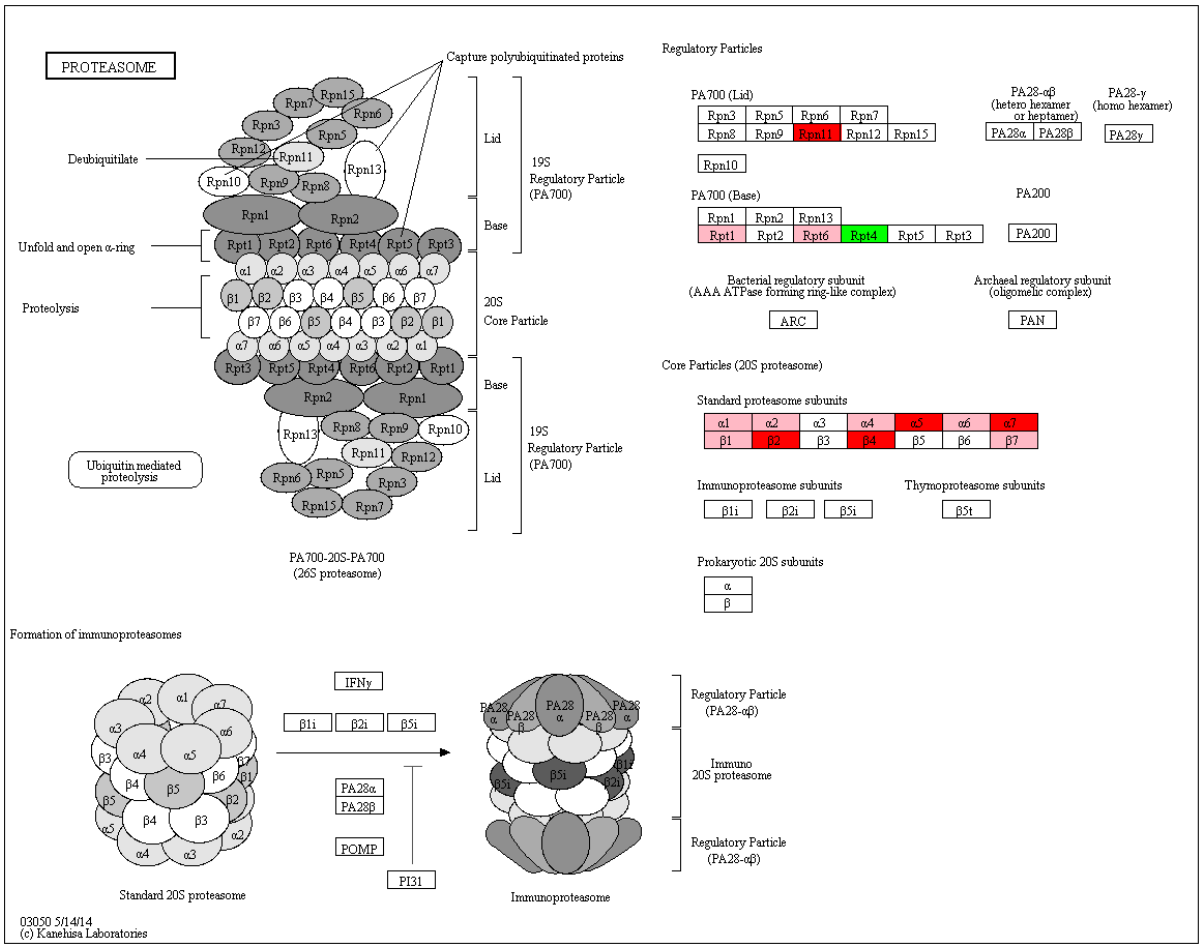

Fig. S5 Regulatory changes on the proteasome, with highlighted up-regulated proteins (green), down-regulated proteins (red) and proteins without fold-change (pink). Labels report EC numbers.

Table S1. Up-regulated proteins with decreasing fold change. Abbreviations. P., plasma membrane, C., cytoplasm; PO., peroxisome; CH., chloroplast; M., mitochondria; V., vacuole; CW., cell wall; CS., cytoskeleton; MM., Membrane; EC., extra cellular; G., Golgi apparatus; V., Vacuole; EM., Endo membrane; ER., endoplasmic reticulum; CHR., Chromosome

UniProt ID	**Locus name**	**description**	**Fold Change**	**Coverage %**	**Unique Peptides**	**Localization**
P93028	AT2G30110	Ubiquitin-activating enzyme E1 1	3.14	1.8	2	P
P34795	AT5G42740	Glucose-6-phosphate isomerase	2.86	3.6	2	C
Q84W89	AT2G42520	DEAD-box ATP-dependent RNA helicase	2.60	7.3	2	PO
Q9SZ30	AT4G26900	Imidazole glycerol phosphate synthase	2.57	3.7	2	CH,
P61837	AT3G61430	Aquaporin PIP1-1	2.57	20	4	M, V, P, CH,
Q9ZVA5	AT1G78860	F9K20.9 protein	2.53	26	2	CW,
Q9C658-2	AT1G26110	Isoform 2 of Protein decapping 5	2.52	5.3	3	C
Q8LF21	AT1G14830	Dynamin-related protein 1C	2.52	3.3	2	C, P, MM,
Q9S7Y7	AT1G68560	Alpha-xylosidase 1	2.44	3.4	4	EC, CW, CH,
Q683F9	AT4G36195	Prolyl carboxypeptidase like protein	2.44	6.3	3	CW, V, P,
Q9LRX8	AT3G24830	60S ribosomal protein L13a-2	2.42	12.6	3	C, R,
Q8L7N0	AT5G16070	At5g16070	2.38	3.4	2	C
Q9XI10	AT1G21680	DPP6 N-terminal domain-like protein	2.30	3.3	3	CW, V, P,
Q56ZI2	AT1G22530	Patellin-2	2.27	26.5	12	P, CH,
Q84WU7	AT3G51330	Aspartyl protease family protein	2.26	5	3	P
Q93W22	AT1G66580	60S ribosomal protein L10-3	2.16	26	1	V, C, R, P,
Q9ZPI1	AT3G11710	Lysine--tRNA ligase	2.08	6.5	4	
Q9C8Y9	AT1G66280	Beta-glucosidase 22	2.08	43.7	17	ER, CH,
P29976	AT4G39980	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	2.07	6.5	1	CH
F4J9K9	AT3G05900	Neurofilament protein-related protein	2.04	34.8	18	C
Q9C522	AT3G06650	ATP-citrate synthase beta chain protein 1	2.04	3.3	2	C, MM
P51418	AT2G34480	60S ribosomal protein L18a-2	2.03	22.5	6	C, R, P,
F4HV16	AT1G47600	Myrosinase 4	2.03	6.5	3	EM
Q8RX87	AT5G20250	Probable galactinol--sucrose galactosyltransferase 6	2.02	4.1	3	CH
Q9SU40	AT4G12420	Monocopper oxidase-like protein SKU5	2.02	5.1	2	EC, CW, P
Q9M8T0	AT3G02880	Probable inactive receptor kinase At3g02880	2.01	14.2	8	CW, P,
Q9FJ62	AT5G55480	Probable glycerophosphoryl diester phosphodiesterase 1	1.99	10.4	7	P
Q94C86	AT3G16850	Glycoside hydrolase family 28 protein / polygalacturonase (Pectinase) family protein	1.98	5.5	2	CW, V
Q9SZ51	AT4G31840	Early nodulin-like protein 15	1.97	22	5	P
O80763	AT1G60420	Probable nucleoredoxin 1	1.97	3.6	2	C
O80988	AT2G26080	Glycine dehydrogenase [decarboxylating]	1.97	2	2	M, CH,
Q9FE65	AT1G69620	60S ribosomal protein L34-2	1.96	17.7	2	C, R, P,
O82762	AT2G25970	F17H15.1/F17H15.1	1.96	18	9	C
Q9SF53	AT3G09500	60S ribosomal protein L35-1	1.95	30	5	Nu, C, R,
Q9LM92	AT1G20580	At1g20580/F2D10_6	1.95	14	2	Nu
Q9LKR3	AT5G28540	Mediator of RNA polymerase II transcription subunit 37a	1.94	45	4	CW, V, P, ER, CH,
Q9STW6	AT4G24280	Heat shock 70 kDa protein 6, chloroplastic	1.90	23	4	M, CH
Q56WK6	AT1G72150	Patellin-1	1.89	24.3	12	EC, V, P, CH,
Q9S791	AT1G70770	AT1G70770 protein	1.87	7.7	4	ER, P

Q7PC86	AT1G15210	ABC transporter G family member 35	1.86	1	2	P, CH,
Q9SR37	AT3G09260	Beta-glucosidase 23	1.83	17	13	V
Q94BT2	AT3G07390	Auxin-induced in root cultures protein 12	1.80	10	3	EC, P
Q9FN52	AT5G23020	Methylthioalkylmalate synthase 3, chloroplastic	1.79	4.4	2	CH
P43287	AT2G37170	Aquaporin PIP2-2	1.79	14.4	3	P, CH,
Q9LXC9	AT5G09650	Soluble inorganic pyrophosphatase 1, chloroplastic	1.79	9.7	4	CH
Q8LA13	AT3G58510	DEAD-box ATP-dependent RNA helicase 11	1.78	9	3	Nu, PO, P,
Q9SR13	AT3G04610	AT3g04610/F7O18_9	1.78	10.8	5	C, N
Q9M9K1	AT3G08590	Probable 2,3-bisphosphoglycerate-independent phosphoglycerate mutase 2	1.77	12.3	4	EC, C
P51422	AT3G55750	60S ribosomal protein L35a-4	1.77	22.5	3	C, R,
F4K0F7	AT5G60640	Protein disulfide-isomerase A1	1.77	29.1	13	M, ER, P, CH
O22126	AT2G45470	Fasciclin-like arabinogalactan protein 8	1.76	23.3	9	EC, CW, P, A
Q8VZH2	AT4G33090	AT4g33090/F4I10_20	1.76	2.3	2	P
Q940M2	AT4G39660	Alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial	1.75	5.0	2	M, P
Q8S9L6	AT4G21410	Cysteine-rich receptor-like protein kinase 29	1.74	4	2	V, P, CH,
Q9C525-2	AT1G66270	Isoform 2 of Beta-glucosidase 21	1.73	37.9	10	V
P16181	AT3G48930	40S ribosomal protein S11-1	1.72	33.7	5	CW, C, R,
B9DG18	AT1G20620	AT1G20620 protein	1.71	10.3	1	EC, CW, M, V, PO, R, P, CH
O04310	AT3G16460	Jacalin-like lectin domain-containing protein	1.68	28	16	C
A8MS66	AT2G29960	Peptidyl-prolyl cis-trans isomerase	1.68	11.5	2	C, ER, MM
Q38882	AT3G15730	Phospholipase D alpha 1	1.67	3.6	3	M, V, P, CH,
F4J110	AT3G63460	Protein transport protein SEC31	1.65	4	4	C, G, MM
Q9SG80	AT3G10740	Alpha-L-arabinofuranosidase 1	1.65	4.6	2	EC, CW,V,
Q8VX13	AT3G54960	Protein disulfide isomerase-like 1-3	1.64	6.9	3	ER, P, CH
B9DHK3	AT5G14540	AT5G14540 protein	1.64	7.7	3	C, N
Q9SZ11	AT4G26690	Probable glycerophosphoryl diester phosphodiesterase 2	1.63	9.7	6	CW, P,
Q96321	AT3G06720	Importin subunit alpha-1	1.63	14.8	8	CW, Nu, C, EM,
F4JBY2	AT3G60750	Transketolase	1.62	10.8	6	CH
Q9LTX9	AT5G49910	Heat shock 70 kDa protein 7,	1.62	22.7	6	EC, CH,
Q43291	AT1G09590	60S ribosomal protein L21-1	1.62	21.9	6	Nu, C, R, CH,
O23006	AT2G17120	LysM domain-containing GPI-anchored protein 2	1.62	19.4	8	P
Q42262	AT4G34670	40S ribosomal protein S3a-2	1.60	20.6	2	CW, Nu, C, R, P,
F4I8B9	AT1G65010	Putative WEB family protein At1g65010	1.59	3.7	1	CH
Q9SRH6	AT3G01290	Hypersensitive-induced response protein 3	1.59	21	6	M, V, P
Q9LFE4	AT5G16730	WEB family protein At5g16730	1.59	6.7	4	CH
P46286	AT2G18020	60S ribosomal protein L8-1	1.59	34.1	8	Nu, V, C, R, P, CH,
O23628	AT3G54560	Histone H2A variant 1	1.59	22	2	CHR,
Q8GYH9	AT3G46280	Protein kinase-like protein	1.58	4	2	ER
Q39043	AT5G42020	Mediator of RNA polymerase II transcription subunit 37f	1.58	49.6	7	CW, Nu, V, ER, P,
O04318	AT3G16390	Nitrile-specifier protein 3	1.58	6.2	2	CH
Q9ZWA8	AT1G03870	Fasciclin-like arabinogalactan protein 9	1.58	15	4	P
Q9S7C0-2	AT1G79930	Isoform 2 of Heat shock 70 kDa protein 14	1.58	11.3	9	P

Q9MAH0	AT1G53310	Phosphoenolpyruvate carboxylase 1	1.57	17.3	13	EC, C
Q9FGK5	AT5G47520	Ras-related protein RABA5a	1.57	10.9	2	P
O82663	AT5G66760	Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1	1.56	7.9	4	CW, M,
F4JWM1	AT5G18380	40S ribosomal protein S16-3	1.56	41.7	6	C, R, CH,
Q42560	AT4G35830	Aconitate hydratase 1	1.54	19.9	9	EC
P92994	AT2G30490	Trans-cinnamate 4-monooxygenase	1.53	11	6	CW, ER, P,
Q9ZQX4	AT4G02620	V-type proton ATPase subunit F	1.53	13.3	2	V, P
O82772	AT3G03640	Probable inactive beta-glucosidase 25	1.53	5.6	3	ER
Q9SZV4	AT4G30010	AT4g30010/F6G3_40	1.52	20	2	M, PL
Q9FN93	AT5G59680	Probable LRR receptor-like serine/threonine-protein kinase At5g59680	1.52	7.5	6	ER
Q9SK27	AT2G25060	Early nodulin-like protein 1	1.52	16.5	3	P
Q9FVT2	AT1G57720	Probable elongation factor 1-gamma 2	1.51	14.3	7	CW, V,
Q9SMT7	AT3G48990	4-coumarate--CoA ligase-like 10	1.51	21	12	EC, CH
Q7Y175	AT4G15410	UBA and UBX domain-containing protein At4g15410	1.51	10.9	4	N
F4IB69	AT1G51850	Leucine-rich repeat protein kinase family protein	1.51	3.8	4	P
O82089	AT3G56240	Copper transport protein CCH	1.50	14.8	5	CH
Q9FXA2	AT1G49760	Polyadenylate-binding protein 8	1.50	21.9	11	C, N
P43286	AT3G53420	Aquaporin PIP2-1	1.50	13.9	4	V, P, CH,
F4JEJ0	AT3G13790	Beta-fructofuranosidase, insoluble isoenzyme CWINV1	1.49	3.6	2	EC, CW,
Q05758	AT3G58610	Ketol-acid reductoisomerase	1.49	7.9	5	EC, CW, M, CH,
Q56XG6	AT5G11170	DEAD-box ATP-dependent RNA helicase 15	1.49	9.6	4	CW, Nu, EC,
O50008	AT5G17920	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	1.48	45.6	20	EC, PO, C, P,
Q9C500	AT1G47200	WPP domain-containing protein 2	1.48	11.7	2	NM, G, P,
Q56ZQ3	AT2G14720	Vacuolar-sorting receptor 4	1.48	17	10	V, G,, P
Q9FLT0	AT5G61780	100 kDa coactivator-like protein	1.48	17	6	C, ER
Q9LV70	AT5G48430	Aspartyl protease family protein	1.47	5.4	2	ER
Q9ASV5	AT4G39690	AT4g39690/T19P19_80	1.47	3.7	2	M
C0LGL4	AT2G28960	Probable LRR receptor-like serine/threonine-protein kinase At2g28960	1.46	2.4	2	ER
Q9ASR1	AT1G56070	At1g56070/T6H22_13	1.46	29	23	Nu, C, P, CH,
Q680P8	AT4G33865	40S ribosomal protein S29	1.46	48	3	C, R,
F4KHD5	AT5G40450	Uncharacterized protein	1.46	12.2	30	P, CH
O22173	AT2G23350	Polyadenylate-binding protein 4	1.45	14	7	C, N
P19456	AT4G30190	ATPase 2, plasma membrane-type	1.45	9.8	8	P
B3H533	AT1G45000	AAA-type ATPase family protein	1.45	6.9	2	CW, Nu, P,
Q93W34	AT4G27000	Polyadenylate-binding protein RBP45C	1.44	16.9	6	C, N
Q9S7L9	AT1G22450	Cytochrome c oxidase subunit 6b-1	1.43	45	8	M, CH,
Q9LXG1	AT5G15200	40S ribosomal protein S9-1	1.43	49.5	13	CW, Nu, C, R, P,
O49299	AT1G23190	Probable phosphoglucomutase	1.43	22.9	12	C, P, CH,
Q9LXB8	AT5G09530	Hydroxyproline-rich glycoprotein family protein	1.43	26.8	4	ER
C0Z361	AT5G56500	Chaperonin 60 subunit beta 3	1.43	17	3	M, CH,
Q9ZVA4	AT1G78850	D-mannose binding lectin protein with Apple-like carbohydrate-binding domain	1.43	41.5	10	EC, CW, P

F4K5C7	AT5G07090	40S ribosomal protein S4-2	1.42	66	17	C, R, P,
P42731	AT4G34110	Polyadenylate-binding protein 2	1.42	26.4	13	C
Q9FJI5	AT5G40760	Glucose-6-phosphate 1-dehydrogenase, cytoplasmic isoform 2	1.41	20.4	12	C
Q9SGE0	AT1G08200	Similar to dihydroflavonol reductase	1.41	5.1	2	EC, C, N
Q94CE5	AT3G22200	Gamma-aminobutyrate transaminase POP2, mitochondrial	1.41	4.8	2	M, P
Q9SRG3	AT1G77510	Protein disulfide isomerase-like 1-2	1.40	32	8	ER, P, CH,
P0DH99	AT1G07940	Elongation factor 1-alpha 1	1.40	36	17	Nu, M, V, P, CH,
F4I035	AT1G48860	3-phosphoshikimate 1-carboxyvinyltransferase	1.40	3.9	2	CH
F4IVR2	AT2G33210	Chaperonin CPN60-like 1	1.40	22	3	M, P, CH,
Q9SIP7	AT2G31610	40S ribosomal protein S3-1	1.40	41.2	3	V, C, R, P, CH,
F4KBN2	AT5G44720	Molybdenum cofactor sulfurase family protein	1.39	11	2	Mi
O48723	AT2G26560	Phospholipase A 2A	1.39	9.3	3	CH, C, MM
Q1H583	AT1G54000	GDSL esterase/lipase At1g54000	1.39	50	15	V, EC, CW, V, P,
Q84VW9	AT3G14940	Phosphoenolpyruvate carboxylase 3	1.39	4.8	1	C
O23654	AT1G78900	V-type proton ATPase catalytic subunit A	1.38	39.6	21	V, CW, V, P, CH,
Q9SQR1	AT3G04010	At3g04010	1.37	3	2	P
Q9M9P3	AT3G03250	Probable UTP--glucose-1-phosphate uridylyltransferase 2	1.37	15.8	6	P
F4KHS2	AT5G59090	Subtilase 4.12	1.37	21.8	14	EC
Q9LF30	AT5G15520	40S ribosomal protein S19-2	1.36	25.9	1	Nu, C, R,
B9DG17	AT1G72370	40S ribosomal protein SA	1.36	39.5	9	C, R, P, CH,
Q9SP02	AT5G58710	Peptidyl-prolyl cis-trans isomerase CYP20-1	1.36	19.1	2	EC, ER, CH,
Q9FM96	AT5G56360	Glucosidase II beta subunit protein PSL4	1.36	11.3	7	EM
Q9FKV0	AT5G44380	Berberine bridge enzyme-like protein	1.36	5.7	3	CW
P22953	AT5G02500	Probable mediator of RNA polymerase II transcription subunit 37e	1.35	45.8	12	EC, CW, Nu, C, R, P, CH,
Q8VZ19	AT1G77940	60S ribosomal protein L30-2	1.35	41	4	C, R,
P17094	AT1G43170	60S ribosomal protein L3-1	1.35	55	25	CW, NU, V, C, R, P,
P11574	AT1G76030	V-type proton ATPase subunit B1	1.35	33.7	7	V, P, CH,
Q8L7E3	AT4G20110	Vacuolar-sorting receptor 7	1.34	23	14	G, P
F4ISU2	AT2G32240	Uncharacterized protein	1.34	45.8	61	P, C
Q29Q26	AT2G17390	Ankyrin repeat-containing 2B	1.34	16.6	5	P
P83483	AT5G08670	ATP synthase subunit beta-1	1.33	56.1	28	CH, P, M
Q8VZT4	AT1G29370	Kinase-related protein	1.33	6.4	5	N
P59223	AT3G60770	40S ribosomal protein S13-1	1.33	41	7	CW, Nu C, R, CH
A8MS28	AT4G15000	60S ribosomal protein L27-3	1.33	30.5	4	C, R,
P53492	AT5G09810	Actin-7	1.33	40	5	CW, Nu, M, CS, P,
Q9M5K5	AT3G16950	Dihydrolipoyl dehydrogenase	1.33	24.6	12	Nu, CH,
Q96291	AT3G11630	2-Cys peroxiredoxin BAS1	1.32	7.9	2	EC, CH
Q9LFW1	AT5G15650	UDP-arabinopyranose mutase 2	1.32	37.5	3	CW, G, R, P,
F4K1Y4	AT5G60980	Nuclear transport factor 2 and RNA recognition motif domain-containing protein	1.32	20.2	9	Intra cellular
Q9LTF2	AT5G52650	40S ribosomal protein S10-3	1.32	37.4	6	CW, C,R
Q9LMK7	AT1G07140	Ran-binding protein 1 homolog a	1.32	37.7	8	NM, M
Q42290	AT3G02090	Probable mitochondrial-processing peptidase subunit beta	1.32	43.9	20	CW, Nu, M

Q96292	AT3G18780	Actin-2	1.32	37.1	4	CS
Q9LX13	AT5G10160	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase-like protein	1.31	13.7	3	CW, CH,
Q9SJ44-3	AT2G36060	Isoform 3 of Ubiquitin-conjugating enzyme E2 variant 1C	1.31	27	1	C,N
Q9FKK7	AT5G57655	Xylose isomerase	1.31	11.3	2	V, ER, P
O65719	AT3G09440	Heat shock 70 kDa protein 3	1.31	42.2	10	EC, CW, V, C, R, P, CH,
Q9SE60	AT3G59970	Methylenetetrahydrofolate reductase 1	1.31	12.5	4	C
Q8RXU5	AT3G60245	60S ribosomal protein L37a-2	1.31	9.8	2	C, R,
Q9SVG4-2	AT4G20830	Isoform 2 of Reticuline oxidase-like protein	1.31	13.7	8	EC, CW, M, V, P
Q9SMU8	AT3G49120	Peroxidase 34	1.31	29.8	6	EC, CW, V, P,
Q84WV1	AT5G26360	Putative chaperonin gamma chain	1.30	10.8	6	C
Q94C59	AT1G30690	Patellin-	1.3	7.2	3	C, P,
O80517	AT2G44790	Uclacyanin-2	1.30	30.2	5	P
A8MR02	AT5G26280	TRAF-like family protein	1.30	33.3	9	CH, MM
Q9SIB9	AT2G05710	Aconitate hydratase 2	1.30	17.7	9	CW, M, P, CH
P20115-2	AT2G44350	Isoform 2 of Citrate synthase 4	1.30	15	6	CW, M, CH,
Q9LST0	AT5G60160	AT5g60160/f15l12_20	1.30	6	3	V, P
Q94A28	AT4G26970	Aconitate hydratase 3	1.30	17.9	14	M, CH
Q9FGT8	AT5G58070	Outer membrane lipoprotein-like	1.30	7.5	2	M, V, ER, P

Table S2. Down-regulated proteins with decreasing fold change. Abbreviations. P., plasma membrane, C., cytoplasm; PO., peroxisome; CH., chloroplast; M., mitochondria; V., vacuole; CW., cell wall; CS., cytoskeleton; MM., Membrane; EC., extra cellular; G., Golgi apparatus; V., Vacuole; EM., Endo membrane; ER., endoplasmic reticulum; CHR., Chromosome

UniProt ID	**Locus name**	**Description**	**Fold Change**	**Coverage %**	**Unique Peptides**	**Localization**
O04922	AT2G31570	Probable glutathione peroxidase 2	0.76	51.5	10	C
O23237	AT4G36430	Peroxidase 49	0.76	7.9	2	EC, CW,
Q9SMW7	AT1G17880	BTF3b-like factor	0.76	26.7	5	N
O49499	AT4G34050	Caffeoyl-CoA O-methyltransferase 1	0.76	28.6	8	C
Q9FNQ2	AT5G61130	Glucan endo-1,3-beta-glucosidase-like protein 2	0.76	11.4	2	CW, P
O22711	AT1G60740	Peroxiredoxin-2D	0.76	6.2	2	P
Q9C787	AT1G69510	Putative uncharacterized protein	0.76	11.7	2	N
Q9LZW6	AT5G01800	AT5g01800/T20L15_70	0.75	11.1	3	EM
P47999	AT2G43750	Cysteine synthase]	0.75	11.8	2	EC, M, CH,
O04331	AT5G40770	Prohibitin-3, mitochondrial	0.75	27.4	7	Nu, M, V, P,
Q9SHC8	AT2G45140	Vesicle-associated protein 1-2	0.75	20.1	5	ER, C, P
A2RVJ8	AT5G10010	At5g10010	0.75	4.9	2	Nu
O23715	AT2G27020	Proteasome subunit alpha type-3	0.75	8.4	2	EC, V, P,
Q940G5	AT4G25900	Aldose 1-epimerase family protein	0.75	10.1	4	CW, P, CW, EC
F4IWI5	AT3G27430	Proteasome subunit beta type	0.74	6.4	2	V
Q9LK88	AT3G27890	NADPH:quinone oxidoreductase	0.74	27.1	5	M, PO, P, CH,
Q9SIP1	AT2G31670	At2g31670	0.74	23.2	6	PO, CH,
O80840	AT2G45790	Phosphomannomutase	0.74	7.7	2	C
A8MR12	AT5G23540	26S proteasome non-ATPase regulatory subunit 14	0.74	5.8	2	C
F4J7G9	AT3G11820	Syntaxin-121	0.74	25.1	6	P
P58684	AT2G39960	Probable signal peptidase complex subunit 2	0.74	25.5	5	ER
Q0WUY5	AT3G07660	Putative uncharacterized protein At3g07660	0.74	3.0	2	N
Q42342	AT5G53560	Cytochrome b5 isoform A	0.73	44.0	7	V, ER, P, CH,
P43333	AT1G09760	U2 small nuclear ribonucleoprotein A'	0.73	9.2	2	Nu, CH,
P17745	AT4G20360	Elongation factor Tu	0.73	8.9	3	EC, Nu, CH,
Q9SUR0	AT4G23670	AT4G23670 protein	0.73	49	8	V
Q9SLJ2	AT1G54410	At1g54410	0.73	23.5	3	CH
Q8LGE7	AT5G18800	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8-B	0.73	14.2	2	CH, M
Q38867	AT3G56070	Peptidyl-prolyl cis-trans isomerase CYP19-3	0.73	14.5	2	C
Q9ZUG4	AT2G05830	Isoform 2 of Methylthioribose-1-phosphate isomerase	0.72	11.3	3	EM
O80837	AT2G45820	Remorin	0.72	52.1	11	P
Q9C505	AT1G69410	Eukaryotic translation initiation factor 5A-3	0.72	36.1	2	C
Q9FMN0	AT5G42890	Putative uncharacterized protein	0.72	28.5	4	PO
Q96253	AT1G51650	ATP synthase subunit epsilon, mitochondrial	0.72	47.1	4	M
Q94K48	AT3G62530	Armadillo/beta-catenin-like repeat-containing protein	0.72	11.8	2	Nu, M, CH
Q93XZ7	AT5G42570	At5g42570	0.72	35.3	8	ER, P
Q9LKA3	AT3G15020	Malate dehydrogenase 2, mitochondrial	0.72	30.8	3	EC, M,
Q39243	AT4G35460	Thioredoxin reductase 1	0.72	16.5	1	M, C, CH,
Q9FWR4	AT1G19570	Isoform 2 of Glutathione S-transferase DHAR1, mitochondrial	0.71	31.1	6	EC, M, V, PO, P, CH,

Q9FPF0	AT3G14990	Isoform 2 of Protein DJ-1 homolog A	0.71	23.3	6	V, P,
O81149	AT1G53850	Proteasome subunit alpha type-5-A	0.71	30.4	7	R,P
Q9SUQ8	AT4G23690	Dirigent protein 6	0.71	15.5	2	EM
Q93W30	AT2G35605	Expressed protein	0.71	28.8	2	N
Q41931	AT1G62380	1-aminocyclopropane-1-carboxylate oxidase 2	0.71	27.8	6	CW
Q9LHH7	AT3G12290	5,10-methylenetetrahydrofolate dehydrogenase/5,10-methenyltetrahydrofolate cyclohydrolase	0.71	8.7	2	CH
Q9SRR8	AT3G07480	2Fe-2S ferredoxin-like protein	0.70	15.1	2	M, CH,
Q9ZRW8	AT1G78380	Glutathione S-transferase U19	0.70	39.8	11	P, CH,
Q9SRY5	AT1G02920	Glutathione S-transferase F7	0.69	55.5	6	V
Q9SDS7	AT1G12840	V-type proton ATPase subunit C	0.69	4.3	2	V, P, CH
Q9C835	AT3G66654	Cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein	0.69	8.5	2	G, P
Q9FE29	AT4G15610	CASP-like protein At4g15610	0.69	10.9	2	P
Q9FGF3	AT5G64430	At5g64430	0.68	8.6	3	CH
O82326	AT2G14880	Expressed protein	0.68	13.5	2	CH
P49200	AT5G62300	40S ribosomal protein S20-1	0.68	44.4	2	CW, C, R
O04311	AT3G16450	AT3G16450 protein	0.68	29	6	EC, N
Q8LBY9	AT5G05960	At5g05960	0.68	35.3	5	EM
F4HUG9	AT1G14210	Ribonuclease T2 family protein	0.68	13.8	3	EM
F4KGV2	AT5G10450	14-3-3-like protein GF14 lambda	0.68	25.6	2	CW, C, P, CH,
O80858	AT2G30930	Expressed protein	0.68	68.9	9	P, CH
Q9LIN3	AT3G26420	AT3g26420/F20C19_14	0.67	13.9	4	C, N
Q9SBA5	AT5G16760	Inositol-tetrakisphosphate 1-kinase 1	0.67	8.2	2	C
B3H4B6	AT4G39200	40S ribosomal protein S25-4	0.67	15.9	2	C, R,
Q96522	AT4G30170	Peroxidase 45	0.67	71.1	17	EC, EM
Q9LK64	AT3G13080	Isoform 2 of ABC transporter C family member 3	0.67	0.9	1	V, EC, V, P,
Q9SI54	AT2G03870	At2g03870	0.66	31.3	6	RNC
F4IZR4	AT3G03100	Putative NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	0.66	12.6	2	M
B9DFR9	AT2G45960	AT2G45960 protein	0.65	13.9	1	V, P, CH,
B3H778	AT4G24830	Argininosuccinate synthase	0.65	8.4	3	CH
Q8VYC5	AT1G18270	Ketose-bisphosphate aldolase class-II-like protein	0.65	2	2	M, N
Q9M2G1	AT3G58550	At3g58550	0.65	13	2	ANC M
P42760	AT1G02930	Glutathione S-transferase F6	0.64	52.9	6	CW, M, V,
Q9FJE8	AT5G59870	Probable histone H2A.7	0.63	15.3	2	CHR, Nu,
Q9LSP5	AT3G17020	AT3g17020/K14A17_14	0.63	11	2	P
Q9FZK4	AT1G27310	F17L21.10	0.63	16.4	2	NM
Q9M011	AT5G01650	Light-inducible protein ATLS1	0.62	22.6	2	CH
Q94A16	AT2G47320	Peptidyl-prolyl cis-trans isomerase CYP21-3	0.62	8.7	2	M
Q9ZPY5	AT2G46540	At2g46540/F11C10.23	0.62	23.1	2	M
O49453	AT4G28440	Uncharacterized protein At4g28440	0.61	18.3	2	C
Q43725	AT3G59760	Cysteine synthase, mitochondrial	0.61	17.4	4	M, CH
Q42338	AT3G48140	AT3G48140 protein	0.60	32.9	3	PO
Q9LU05	AT5G44610	At5g44610	0.60	5.9	2	P
P24704	AT1G08830	Superoxide dismutase [Cu-Zn] 1	0.60	31.6	5	C

Q9ZVF1	AT2G01540	Calcium-dependent lipid-binding domain-containing protein	0.58	11.7	2	V, P,
Q9C6U3	AT3G08030	Putative uncharacterized protein T8G24.2 (Fragment)	0.58	8.7	2	CW
Q3E902	AT5G27700	40S ribosomal protein S21-2	0.57	24.4	3	C, R, P, CH,
Q9FMA8	AT5G38940	Germin-like protein subfamily 1 member 11	0.57	30.5	3	EC, CW
Q9SFF9	AT3G05950	Germin-like protein subfamily 1 member 7	0.57	24.9	4	EC, EM
Q9SF20	AT3G11780	F26K24.7 protein	0.57	21.6	3	V, P,
Q9FMA9	AT5G38930	Germin-like protein subfamily 1 member 10	0.57	10.7	1	EC, CW,
P31166	AT1G27450	Isoform 2 of Adenine phosphoribosyltransferase 1	0.57	21.9	3	CW, C, P, CH,
Q9FY99	AT5G13110	Glucose-6-phosphate 1-dehydrogenase 2	0.55	5	2	CH
O49006	AT3G14310	Pectinesterase/pectinesterase inhibitor 3	0.55	4.7	2	EC, CW, P,
Q8L8Y0	AT1G58380	40S ribosomal protein S2-1	0.54	46.5	3	C, R, P, CH,
O23629	AT3G45980	Histone H2B.6	0.52	29.3	1	CHR, Nu,
Q9ZVF2	AT2G01530	MLP-like protein 329	0.52	35.1	3	N
Q681K2	AT2G41475	Embryo-specific protein 3, (ATS3)	0.51	11.7	2	EM
P56757	ATCG00120	ATP synthase subunit alpha	0.46	3	1	CH, M
P40283	AT5G59910	Histone H2B.11	0.46	29.3	1	CHR, Nu,
Q9FIC6	AT5G39150	Germin-like protein subfamily 1 member 17	0.45	15.4	2	EC, EM
P43296	AT4G39090	Cysteine proteinase RD19a	0.44	14	4	V
O24633	AT4G14800	Proteasome subunit beta type-2-B	0.43	14	2	V
Q9LHQ5	AT3G20670	Probable histone H2A.2	0.43	26.5	2	CHR, Nu,
Q8LAJ9	AT1G22520	At1g22520	0.40	23.2	2	M
B3H5Q0	AT5G39190	Germin-like protein subfamily 1 member 20	0.36	13	2	EC
P0C1T5	AT5G09978	Elicitor peptide 7	0.35	22.3	2	M
Q9C681	AT1G51060	Probable histone H2A.1	0.35	26.5	2	CW, Nu,
Q56W16	AT1G52370	AT1G52370 protein	0.17	5.2	2	R
Q8GYZ3	AT1G19130	Putative uncharacterized protein At1g19130/F14D16_18	0.17	10.4	2	C
Q9FX82	AT1G16690	Enhancer of polycomb-like transcription factor protein	0	2.7	1	N
Q9S7A0	AT3G03910	Probable glutamate dehydrogenase 3	0	8.8	1	M

4 Manuscript II

Effects of humic substances and indole-3-acetic acid on Arabidopsis sugar and amino acid metabolic profile

Giovanni Battista Conselvan[a], David Fuentes[b], Andrew Merchant[b], Cristina Peggion[c], Ornella Francioso[d], Paolo Carletti[a,*]

[a] Dipartimento di Agronomia, Animali, Alimenti, Risorse Naturali e Ambiente (DAFNAE), Università di Padova, Viale dell'Università 16, 35020 Legnaro (Padova), Italy

[b] School of Life and Environmental Sciences, The University of Sydney, 380 Werombi Road, Camden, NSW 2570, Australia

[c] Department of Chemical Sciences, University of Padova, 35131 Padova, Italy

[d] Dipartimento di Scienze Agrarie, Università di Bologna, Viale Fanin 44, 40127 Bologna, Italy

[*] corresponding author

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Abstract

Background and aim Humic substances (HS) are known to influence plant physiological processes, enhancing crop yield, plant growth and nutrient uptake. The present study sought to gain a better understanding of the specific effects of HS application on the abundance of metabolites in plant tissues, using mass spectrometry analyses.

Methods Arabidopsis thaliana plants, grown in hydroponic conditions, were treated for 8 h with indole-3-acetic acid (IAA), HS from International Humic Substances Society (IHSS) and HS from earthworm *faeces* (EF), respectively. Humic substances structural characteristics were analyzed by ^{1}H NMR an FT-IR spectroscopies. Root and leaf free amino acids, sugar alcohols and carbohydrate contents, and leaf amino acids from protein hydrolysis were identified and quantified by gas chromatography-mass spectrometry (GC/MS), and liquid chromatography-mass spectrometry (LC/MS). Canonical discriminant analysis (CDA) was used to evaluate the influence of the treatments on the studied parameters.

Results EF treatment had the highest influence on metabolite profiles compared to the control, IAA and IHSS. CDA analysis highlighted a clear distinction between EF and IHSS plant physiological responses, depending on the different chemical and structural properties of the HS. IAA-treated plants showed not significant difference from the control.

Conclusions A better understanding of the specific effects of different HS, also related to their chemical characteristics, might serve as a basis for the identification of marker compounds for HS bioactivity.

Keywords

Humic substances, metabolomics, biostimulant, auxin-like activity, FT-IR, ^{1}H NMR

Introduction

Humic substances (HS) are a mixture of heterogeneous organic compounds resulting from the microbial decomposition and chemical degradation of plant and animal residue in the soil (Carletti et al. 2008; Nardi et al. 2009). They are present in both terrestrial and aquatic environments, being the most abundant component of organic matter on earth (Nardi et al. 2002; Salma et al. 2010). Thanks to their oxygenate functional groups (e.g. carboxylic and phenolic hydroxyl), HS are one of the most reactive fractions of organic carbon (Nardi et al. 2017). HS have a supramolecular structure, resulting from the association of small molecules by hydrogen bonds and dispersive hydrophobic interactions (van der Waals, π-π, and CH-π bondings) (Baigorri et al. 2007; Piccolo 2001). Polar groups constitute the external layer, whereas plant macromolecules make up the hydrophobic interior domain. HS can be extracted from different humified organic matter (e.g. from soil compost, vermicompost, or mineral deposits such as leonardite or coal) (du Jardin 2012). Humic substances influence soil fertility, by improving the structure, porosity and supply of nutrients (Bronick and Lal 2005; Nardi et al. 2009) as well as influencing plant physiological processes that enhance crop yield, plant growth and nutrient uptake (Nardi et al. 2002). These properties allow HS to be considered as a class of biostimulants (Calvo et al. 2014). Biostimulants are substances that at low concentrations, increase plant nutrient uptake, nutrient efficiency, resistance to biotic and abiotic stress, and crop quality (European Biostimulant Industry Council 2013).

Despite these properties, little is known regarding the mechanistic effects of HS. HS are known to have a direct influence on lateral root induction, root hair production, and H^+-ATPase activity, thanks to their hormone-like activity (Pizzeghello et al. 2015), which could partly be attributed to small bioactive molecules (e.g. auxin) present within the HS supramolecular structure (Canellas and Olivares 2014; Canellas et al. 2002; Mora et al. 2014; Olaetxea et al. 2015). Zandonadi et al. (2007) demonstrated that HS induce nitric oxide (NO) production in sites of lateral root emergence. NO plays a role in different plant physiological processes, including root and root hair formation and elongation (Lombardo and Lamattina 2012). Plant responses to HS in terms of H^+-ATPase activity induce a decrease in cell wall pH, triggering the activity of enzymes and proteins involved in cell-wall loosening and extension growth (Hager 2003).

Moreover, the activation of H^+-ATPase enzyme enhances the electrochemical proton gradient that drives ion transport across cell membranes via secondary transport systems, improving plant nutrient uptake (Baldotto et al. 2009; Morsomme and Boutry 2000).

HS are reported to induce changes in both primary and secondary metabolism. Nardi et al. (2007) demonstrated that enzymes involved in glycolysis and tricarboxylic acid cycle (TCA) were upregulated after maize plants were treated with 1 mg C L^{-1} (1 mg carbon L^{-1}) of HS and also enhance also N uptake/assimilation and N metabolism. Decreasing pH at the root surface, HS facilitate H^+/NO_3^- symport (Nardi et al. 2000) and stimulate nitrate uptake, transport and the activity of enzymes involved in nitrogen metabolism (Quaggiotti et al. 2004; Vaccaro et al. 2015). Moreover, phenylpropanoid metabolism and enzymes linked to cell protection have been found to be positively affected by treatment with HS (Garcia et al. 2016).

In the last decade, new molecular approaches (genomics, transcriptomics, and proteomics) have been used to characterize the effects of HS on plant metabolism. Trevisan et al. (2011), performed transcriptomic analysis and Gene Ontology classification and detected that HS regulate a large number of genes involved in developmental and metabolic processes, transcription regulation, and RNA metabolism. In *Brassica napus,* after three days of treatment, HS significantly influenced the expression of more than 300 genes involved in the major metabolic plant functions: respiration and photosynthesis, general cell metabolism, fatty acids, nitrogen/sulphur, phytohormones, plant development, senescence, responses to stress, and transport of ions and water (Jannin et al. 2012). Carletti et al. (2008) reported that 42 plasma membrane proteins related to energy, metabolism, and cellular transport were differentially expressed after the treatment of maize roots with HS. In treated soybean *Bradyrhizobium liaoningense* $CCBAU_{05525}$, water-soluble humic materials influenced the expression of proteins involved in nitrogen and carbon metabolism, nucleic acid metabolism, signaling, energy production, and transmembrane transports (Gao et al. 2015). Metabolomic studies or studies targeting the effects of HS on subsets of plant metabolism are still scarce. Nevertheless, using these broad, quantitative profiling techniques, it is possible to better understand the metabolic and biochemical status of an organism, and to monitor changes in a biological system (Griffin and Shockcor 2004; Ren et al. 2015). In a rare example of metabolomics techniques applied to studies on HS responses, Marino et al. (2013) demonstrated through ^1H HR-MAS NMR analyses, that pear and quince calli treated with humic acids had a higher production of asparagine compared to the control.

Amino acids are organic compounds with an essential role in biological processes, being the

building blocks of peptides and proteins (Gu et al. 2015). Carbohydrates, produced during the photosynthesis, are a source of energy for the plant growth and a source of carbon skeletons for organic compounds (Trouvelot et al. 2014). They have also a pivotal function as signal molecules necessary for the coordination of the plant metabolism with growth, development, and responses to biotic and abiotic stresses (Rolland et al. 2006). In addition, both cyclic and acyclic sugar alcohols are common in the soluble components of plant tissues. Sugar alcohols are commonly involved in the response to tolerance to low temperatures, drought, or salt-stress (Moing 2000). The combined detection and quantification of amino acids, carbohydrates, and sugar alcohols are useful tools to study metabolic responses of organisms to external or internal perturbations (Yanes et al. 2011).

The present study sought to gain a better understanding of the specific effects of HS application on the abundance of metabolites in plant tissues through mass spectrometry analyses. To address this aim and isolate HS specific effects from that of more general growth modulating hormones, Arabidopsis *thaliana* plants were treated for eight hours with indole-3-acetic acid (IAA), HS from International Humic Substances Society (IHSS), and HS from earthworm *faeces* (EF). The tested humic substances were characterized by means of FT-IR and ^{1}H-NMR spectroscopies. Free amino acids, amino acids from proteins hydrolysis, sugar alcohols, and carbohydrates profiles of each treatment were identified by gas chromatography-mass spectrometry (GC/MS), and liquid chromatography-mass spectrometry (LC/MS) techniques.

Materials and methods

Preparation of Humic Extract from earthworm faeces

The *faeces* of *Nicodrilus* [=*Allolobophora* (Eisen)=*Aporrectodea* (Oerley)] *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) (Minelli et al. 1995) were collected from the Ah horizon of an uncultivated couchgrass, *Agropyron repens* L., grown in soils classified as Calcaric Cambisol (CMc-F.A.O. classification) (FAO-UNESCO 1997). Earthworm culture conditions, HS extraction, and extract purification were conducted as reported in (Carletti et al. 2008). HS extraction and purification was performed with 0.1 N KOH. The extract was desalted by using 14 kDa cut-off dialysis Visking (Medicell, London, UK) tubing against distilled water. Subsequently, the extract was desalted on ion exchange Amberlite IR-120 (H^+ form), assessed for organic carbon content, and lyophilized before conducting the following analyses. Elliott Soil Humic Acid Standard (code 1S102H) (Saint Paul, MN; USA) purchased by the International *Humic* Substances Society (*IHSS*) was used in this work as a reference.

Chemical and structural properties (FT-IR, and ^{1}H NMR)

Elemental analysis (C, H, N, O) was carried out using an elemental analyser (CHNS-O mod. EA 1110) as per Conselvan et al. (2017). The oxygen has been evaluated by difference. Elemental analysis for IHSS was taken from the IHSS website (http://humic-substances.org). All analyses were performed in triplicate.

IR spectra were recorded with a Bruker ALFA FT-IR Spectrophotometer (Bruker, Ettlingen, Germany) equipped with a single reflection diamond ATR sampling module. The spectra were collected from 4000 to 400 cm^{-1} and averaged over 64 scans (resolution 4 cm^{-1}). The spectral data were processed with Grams/386 spectroscopic software (Galactic Industries, Salem, NH, USA).

^{1}H NMR spectra were carried out with a Bruker Avance 600 MHz instrument at 298 K. The samples (15 mg) were dissolved in 600 µL of DMSO-d_6 and the spectra were acquired using 512 scans. DMSO-d_6 is valid solvent for humic substances measurements because hydrogen bonds are dissipated and their aggregation is avoided. The partially deuterated solvent (DMSO) was used as the internal reference (chemical shift of the residual peak, 2.5 ppm). Spectra were acquired using Bruker standard pulse sequences.

The CPMG (Carr-Purcell-Meiboom-Gill) pulse train is a fundamental component of pulse sequences used for the measurement of dynamic processes by NMR spectroscopy. This pulse

sequence suppresses the effect of diffusion. This is very beneficial and can be used to enhance S/N, and reduce experimental time.

Outcomes are derived from relative integrated areas of typical regions in the ^{1}H-NMR spectrum (Ferrari et al. 2011). Integrals were evaluated by AMIX-Viewer Bruker on the following integration areas: amide group in peptides and aromatics (9.46–7.63 ppm); −CH=CH−, aromatic amino acids, lignin (7.53–6.52 ppm); carbohydrates, β protons in peptides, lignin, ethers (6.40–2.60 ppm); side chain protons in amino acids (2.34–2.04 ppm); $-CH_2-$, chains of lipids, waxes and cuticles (2.04–0.96 ppm); terminal CH_3 groups (0.96–0 ppm). Each integrated area is the average value on three independent calculations, standard error is <5%.

Plant growth and treatment conditions

Arabidopsis (*A. thaliana*) (ecotype Columbia) plants were grown hydroponically in a growth chamber as described previously in Destro et al. (2011). After vernalisation for two days at 4 °C, Arabidopsis *thaliana* wild-type seeds were transferred to the hydroponic system. For the first 30 days seedlings were grown in pools containing half force of Murashige and Skoog basal salt mixture (Sigma-Aldrich) and Murashige and Skoog vitamin solution 1000x (Sigma-Aldrich) (Murashige and Skoog 1962), and for the last 30 days they were grown with full force (4.3 g L^{-1} of salt mixture and 1 mL L^{-1} of vitamin solution). The medium was changed every 3 days. Growth condition were: 10 hours of light at 21 °C, 1 hour of half-light intensity with a decrease of temperature from 21 °C to 18 °C, 12 hours of dark at 18°C, 1 hour of half-light intensity with an increase of temperature from 18 °C to 21 °C, and constant 60% relative humidity. The irradiance at the plant level was of 100-200 μmol photons m^{-2} s^{-1}. After 60 days of pre-cultivation, plants were partitioned in four hydroponic system batches: one, containing Murashige and Skoog solution, was kept as control (CT); one containing Murashige and Skoog solution and 34 nM of indole-3-acetic acid (IAA); one containing Murashige and Skoog solution and 1 mg C L^{-1} of HS from International Humic Substances Society (IHSS); and one containing Murashige and Skoog solution and 1 mg C L^{-1} of HS from earthworm *faeces* (EF) (Trevisan et al. 2010). The concentration of IAA was corresponding to the content of IAA measured in the humic substances extracted from earthworm *faeces* (EF) (34 ± 0.31 nM) as reported in Trevisan et al. (2010). The EF and IHSS concentrations were chosen based on previous studies (Carletti et al. 2008; Nardi et al. 2000; Trevisan et al. 2011).

Each hydroponic system batch was replicated 4 times. After 8 hours of treatment, one plant root sample (approx. 200 mg) and two plant leave samples (approx. 600 mg for each one) were collected for each batch, weighted, snap frozen in liquid nitrogen, and freeze dried. Summarizing, for each treatment 8 leaf and 4 root biological replicates were obtained.

Free amino acids, sugar alcohols, and carbohydrates extraction

The extraction procedure has been described earlier (Merchant et al. 2006). Approximately 40 mg of dried leaf or root material were weighed into a 2 mL screw-cap micro-tube. 1 mL of methanol/chloroform/water (12:5:3) was added and incubated at 75 °C for 30 min. The water fraction of the extraction mixture consisted of a 0.1 % solution of penta-erythritol internal standard. After cooling, samples were centrifuged (11,400 g for 3 min) and 800 mL of the supernatant removed and placed into a clean 2 mL round bottomed micro-tube. A further 200 mL of chloroform and 500 mL of ultrapure water were added to facilitate the separation of phases. The mixture was vortexed for 30 seconds, centrifuged (11,400 g for 3 min) and left to stand for 15 min to allow phase separation. 700 mL of the upper phase (the water–methanol soluble fraction) were transferred to a clean 1.5 mL micro-tube.

Protein hydrolysis

Approximately 20 mg of dried leaf material were weighted and placed into a Vacuum Hydrolysis Tube (Thermo Scientific, Waltham, USA). 1 mL of 6 N HCl was pipetted into the tube, a screw was added and vacuum was created with a pump. Tubes were left for 24 h at 110 °C on a block heater (Thermo Scientific, Waltham, USA). After cooling, 3 mL of ultrapure water were added into each tube, mixed, and 2 mL of each solution were pipetted into a clean 2 mL micro-tube. Samples were then frozen at -20 °C until analysis (Leaf et al. 1958). Thryptophan is easily degraded by acid hydrolysis in 6 N HCl and therefore it cannot be detected.

Analysis of carbohydrates and sugar alcohols

For the carbohydrates and sugar alcohols analyses, gas chromatography (GC) techniques used by Merchant et al. (2006) were followed accordingly. 50 µL of dried extract were suspended in 450 µL anhydrous pyridine to which a solution of 1:10 ratio mixture of trimethylchloroacetamide (TMCS) and bis-trimethylsilyl-trifluroavetamide (BSTFA) was added for derivatization. Samples were incubated for 35 min at 75 °C and analyzed by GC-

MS within 24h. The analysis was carried out on an Agilent 6890 Gas Chromatograph with QQQ 7000 Mass selective detector (Agilent Technologies, Santa Clara, United States). Samples were injected in a split splitless injector at 300 °C with a 20:1 split injection onto a HP-5 column (30 m, 0.25 mm ID, 0.25 µm film thickness) with helium carrier gas at a constant flow of 1 mL/min. The temperature program had an initial oven temperature set of 60 °C for 2 min, ramping to 220 °C at 10 °C min^{-1} for 5 min then to 300 °C at 10 °C min^{-1} for 5 min. GC-MS results were identified based on retention times relative to standards and extracted ions. Peak areas were integrated and their relative quantities were calculated by the Mass Hunter software (version B.07.01, Agilent Technologies), used for peak integration.

Analysis of amino acids

LC-MS analysis of the amino acids extract was carried out on a 1290 Infinity LC system (Agilent Technologies) coupled to a 6520 QTOF Mass selective detector (Agilent Technologies). 3.5 uL of sample was injected into a Zorbax SB-C18 column (2.1 x 150 mm, 3.5 µm) and separation was achieved by gradient elution with water and methanol (98 : 2 % starting mix). The QTOF was tuned to operate at the low mass range <1700 AMU and data acquisition was done in scan mode (60-1000 m/z) and ionization was positive ion mode. LC-MS results were identified based on their retention times relative to standards as well as their formula mass. Peak areas were integrated and their relative quantities were calculated by the Mass Hunter software (version B.07.01, by Agilent).

Statistical analysis

The data represent the means of measurements from four different batches per treatment. For each measurement, the average ± standard error is reported. Analysis of variance (one-way ANOVA) was performed using the SPSS 23 (IBM Corp) software with type of treatment as factor, and was followed by pairwise post hoc analyses (Duncan test) to determine which means differed significantly at $P < 0.05$. Levene and Mauchly's tests were applied to check homoscedasticity and sphericity, respectively, to ensure that assumptions of the model were met as recommended in Field (2013).

Data were further processed with the SAS software 9.1 (SAS institute). Canonical discriminant analysis (CDA) was performed to determine which set of variables best predicts group membership and to visualize the data by condensing the multiple treatment variables onto one or more axes. The analysis seeks to derive a reduced set of discriminant (canonical)

functions that best describe the separation of the studied plots, which in our case were the treatment of Arabidopsis plants. The method also applies a stepwise procedure in order to select the set of variables that has the highest correlation with the factor and assesses the relative importance of each independent variable. The consistency and overall robustness of the separation was evaluated by the Mahalanobis quadratic distance, an index that measures the squared distances between classes, i.e. between the centroids of each cloud, thus reflecting the actual separation between the treatments (Carletti et al. 2009).

Results

Chemical characterization

The chemical characterizations of IHSS and EF are reported in Table 1. The elemental composition was very similar between samples, but IHSS had a higher content of C (58.13%) and of H (3.68%), meanwhile EF had a higher content of O (36.50%) and N (4.41%).

Table 1 Elemental composition (%) of humic substances from International Humic Substances Society (IHSS) and from earthworm *faeces* (EF). Data are mean ± SE, *n*=3

Humic substance	H2O	Ash	C	H	O	N	S	P
IHSS*	8.2	0.88	58.13	3.68	34.08	4.14	0.44	0.24
EF	-	-	55.97 ± 0.17	3.12 ± 0.10	36.5 ± 0.12	4.41 ± 0.16	-	-

* Data of the elemental analysis for IHSS were taken from the IHSS website (http://humic-substances.org)

FTIR spectra

The humic acids spectra were characterized by similar functional groups (Fig. 1). A broad band centered at about 3270 $cm^{-1}$was assigned to the OH stretching vibrations in alcohols, phenols and carboxylic acids. The aliphatic groups were confirmed by the presence of two peaks at around 2920-40 and around 2840 cm^{-1}, due to the asymmetrical and symmetrical stretching of methylene ($-CH_2-$) groups, respectively (Giovanela et al. 2004). The carboxyl groups were established by the band at about 1708-20 cm^{-1}, assigned to the C=O stretching vibration. It was stronger in IHSS than EF. Other bands at around 1610 cm^{-1} can be mainly due to C=C stretching of aromatic rings (Pizzeghello et al. 2015; Tinti et al. 2015) and aromatic carboxylic acids. The region between 1480 and 1300 cm^{-1} was assigned to CH_2 and CH_3 bending, and C-OH deformation of COOH, and COO^- symmetric stretching. The band at around 1220-40 cm^{-1} may be due to C–O stretch and O–H deformation of COOH, phenols and unsaturated ethers. Usually, the region between 1100 - 1000 cm^{-1} is attributed to C–O stretch of alcohols and carbohydrates.

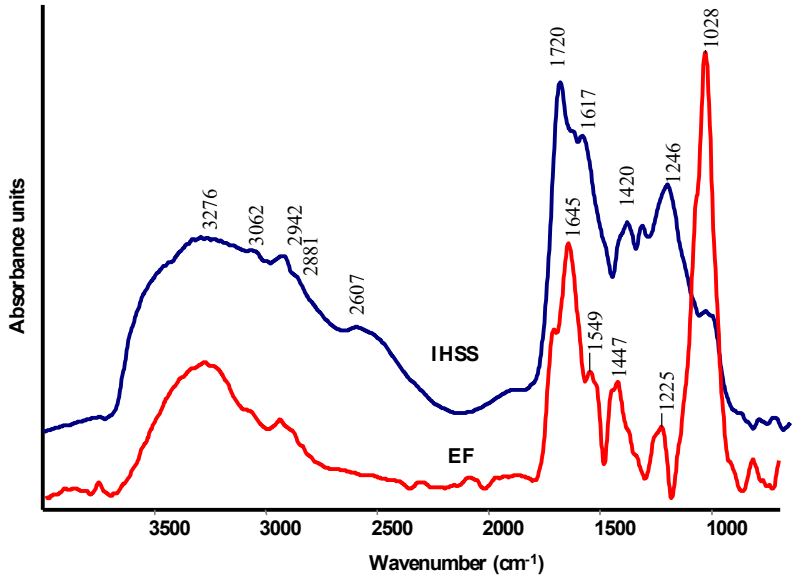

Fig. 1 FT-IR spectra of humic substances from International Humic Substances Society (IHSS), blue line, and from earthworm *faeces* (EF), red line.

Concerning the EF, its spectrum was characterized by the typical bands associated with amide I (1645 cm^{-1}) and amide II (1540 cm^{-1}) (Carletti et al. 2010). However we cannot exclude the contribution of C=C stretch in aromatic rings. In addition, the content of carboxylic groups was not so relevant as that of IHSS because of i) the lack of the band at 2607 cm^{-1}, attributed to hydrogen-bond of carboxylic acids dimers (Bellamy 1975), ii) the appearance of a weak shoulder at 1708 cm^{-1} and iii) the low intensity of the band at 1226 cm^{-1}. This feature highlighted the weak acidic character of EF. In this case, the strong band at 1028 cm^{-1} is attributed to alumino-silicate (Madejova 2003; Tinti et al. 2015) not completely removed during the extraction process.

One-Dimensional ^{1}H NMR Spectra

The spectra are characterized by typical resonance as described in previous paper by Simpson et al. (2001) and Ferrari et al. (2011). As shown in Fig. 2 different regions corresponding to functional groups/molecular moieties can be seen at: 9.00–7.60 ppm are due to protons in peptides and aromatics; 7.60–6.59 ppm are assigned to −CH=CH− and aromatic amino acids, lignin; 5.55–3.00 ppm are characteristic of protons in carbohydrates, β protons in peptides, lignin, ethers; 2.35–2.07 ppm are attributed to side chain protons in amino acids; 2.05–0.97 ppm are assigned to protons in $-CH_2-$, chains of lipids, waxes and cuticles; 0.96–0 ppm are proton in terminal $-CH_3$ groups. In more detail, the IHSS spectrum was dominated by the large contribution of long aliphatic chains $(-CH_2)_n$ and terminal methyl groups as also

supported by IR spectrum (Fig. 1). A small contribution appeared from the α-proton in peptides (~4–4.5 ppm) and sugar-like components (3-4.4 ppm). In contrast, the aromatic signal (~7.60–6.59 ppm) was considerable and in particular, the broad signal from ~7.5–8.5 ppm may mainly include the presence of condensed aromatic rings. The most intense peak was apparent at approximately 1.9 ppm and the other peaks were observed at approximately at 2.1, 3.8, 5.8 ppm. The presence of well-resolved peaks in the spectrum suggests that some simple chemical compounds are incorporated into and/or coexist in IHSS.

EF spectrum was dominated by strong signals of long aliphatic chains in (-CH_2) and in terminal methyl groups, and by a prominent signal of sugar like (3-4.4 ppm) and aromatic (~7.60–6.59 ppm) compounds. However it showed less prominent peaks with respect to IHSS. The integration of the various chemical shift areas of IHSS and EF are given in Table 2. Aromatics and cumulative aliphatic protons accounted for 12.13% and 45.15 % in IHSS and 11.28% and 34.94 % in EF, respectively. Protons in carbohydrates and peptides accounted for 28.27% in IHSS and 45.58% in EF, respectively.

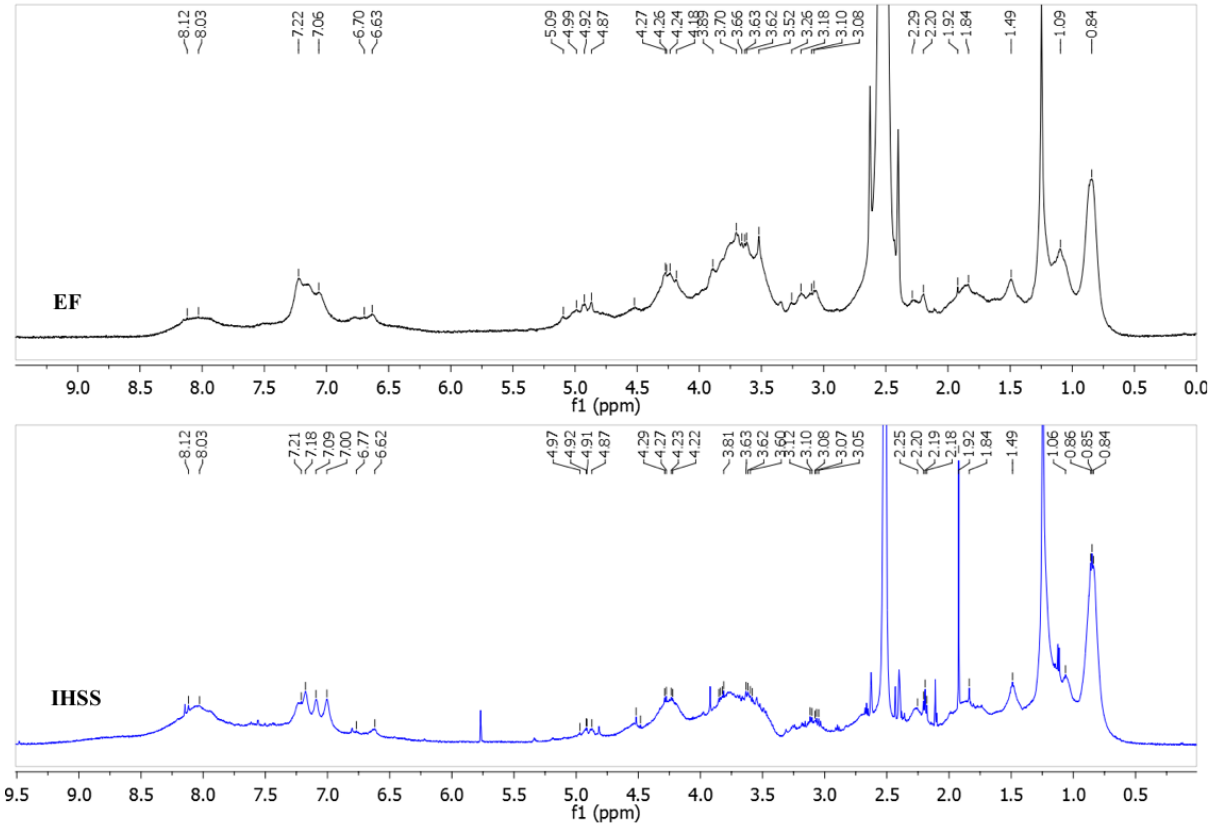

Fig. 2 ^{1}H NMR spectra of (from bottom to top) humic substances extracted from International Humic Substances Society (IHSS), blu line, and earthworm *faeces* (EF), black line. Spectra were registered at 298 K in DMSO-d6.

Table 2 Integrated areas of typical resonances expressed as percentage and calculated on HS from IHSS (IHSS), and HS from earthworm *faeces* (EF) ^{1}H NMR spectra. Integrals were evaluated by AMIX-Viewer Bruker and each integrated area is the average value on three independent calculations, standard error is <5%.

Humic substance	Terminal CH_3 groups	$-CH_2-$, chains of lipids, waxes and cuticles	Side chain protons in amino acids	Carbohydrates, β protons in peptides, lignin, ethers	−CH=CH−, aromatic amino acids, lignin	Amide group in peptides and aromatics
	0-0.96 ppm	0.97-2.05 ppm	2.07–2.35 ppm	3.00-5.55 ppm	6.59-7.60 ppm	7.60-9.00 ppm
IHSS	11.79	34.36	4.22	28.27	12.13	10.47
EF	9.30	26.64	3.97	45.58	11.28	3.24

MCW extracted metabolites

Out of the 40 metabolites investigated, 23 were found in roots and 20 in leaves of treated and untreated plants, respectively. In roots five metabolites were carbohydrates, one was sugar alcohol, and 17 were amino acids. The treatment of Arabidopsis plants with EF and IHSS considerably influenced metabolite contents (Table 3) in roots ($P \leq 0.05$). EF induced a significant reduction ($P \leq 0.05$) of all detected root carbohydrates (ranging from - 48 % to - 54 %) and of myo-inositol (- 48 %) compared to the control. Also for 12 of 17 detected root amino acids similar reduction was observed ($P \leq 0.05$), ranging from - 40 % compared to control for proline, although not significant, and - 54 % for glutamine. Plant treated with IHSS had the lowest production of mannose (38 ± 0 μg g^{-1} dw) and proline (163 ± 38 μg g^{-1} dw) (Table 3) in roots ($P \leq 0.05$).

In leaves 5 carbohydrates, 1 sugar alcohol, and 14 amino acids were identified (Table 3). For carbohydrates, IAA and IHSS treatment showed a significant increase of + 16 % and + 22 % ($P \leq 0.05$) of fructose respectively compared to the control. Meanwhile sucrose content was 40 % and 22 % lower for plants treated with EF and IHSS ($P \leq 0.05$) respectively. EF treatment resulted in a significant reduction of leaf sugar alcohol (- 23 %), and of leaf glutamine, asparagine, threonine, glutamic acid, and proline production (respectively - 17 %, - 19 %, - 20 %, - 21 %, and – 10 % compared to the control) ($P \leq 0.05$).

Amino acids from proteins hydrolysis

After the hydrolysis of leaf proteins, 13 amino acids were found in treated and control samples (Table 4). Plants treated with IAA had a significant lower concentration of serine, alanine, and glutamic acids (Table 4) ($P \leq 0.05$). In samples of IHSS treated plants it was detected a significant higher concentration of arginine, aspartic acid, proline, and phenylalanine ($P \leq 0.05$), but a significant decrease of glycine, serine, alanine, and glutamic

acid ($P \leq 0.05$). EF induced a significant increment of arginine, proline, leucine, and phenylalanine ($P \leq 0.05$), with the highest influence for phenylalanine (+ 28 % compared to untreated), and a significant reduction of glycine, serine, alanine, and glutamic acid concentrations (- 20 %, -33 %, -36 %, and -31%, respectively $P \leq 0.05$).

Table 3 Average values (mean ± SE) of the identified carbohydrates, sugar alcohol, and free amino acids in roots and leaves of control plants (CT), plants treated with auxin (IAA), HS from IHSS (IHSS), and HS from earthworm faeces (EF). Values are expressed in µg g^{-1} of dry matter. Letters indicate significant differences among treatments ($P \leq 0.05$) based on Duncan post hoc tests.

	Metabolite	Roots				Leaves			
		CT	IAA	IHSS	EF	CT	IAA	IHSS	EF
Carbohydrates	Fructose	510 ± 74^{a}	478 ± 121^{ab}	340 ± 54^{ab}	238 ± 34^{b}	276 ± 9^{b}	321 ± 14^{a}	338 ± 20^{a}	294 ± 4^{ab}
	Glucose 1	523 ± 61^{a}	488 ± 102^{a}	373 ± 46^{ab}	250 ± 31^{b}	1839 ± 342	1516 ± 204	1549 ± 237	1405 ± 172
	Glucose 2	555 ± 57^{a}	528 ± 98^{a}	395 ± 39^{ab}	265 ± 35^{b}	2753 ± 521	2254 ± 330	2303 ± 376	2081 ± 287
	Sucrose	12038 ± 2320^{ab}	14413 ± 1456^{a}	12768 ± 3756^{ab}	6305 ± 1304^{b}	22400 ± 1647^{a}	20391 ± 1528^{ab}	17694 ± 599^{b}	13546 ± 1217^{c}
	Mannose	365 ± 156^{a}	415 ± 125^{a}	38 ± 0^{b}	33 ± 0^{b}	428 ± 68	343 ± 54	429 ± 37	388 ± 23
Sugar alcohol	Myo Inositol	398 ± 51^{a}	373 ± 84^{a}	275 ± 39^{ab}	190 ± 23^{b}	664 ± 57^{a}	583 ± 37^{ab}	566 ± 31^{ab}	509 ± 31^{b}
Amino acids	Glutamine	18211 ± 1763^{a}	21210 ± 4782^{a}	20769 ± 3530^{a}	8531 ± 491^{b}	2247 ± 72^{a}	2146 ± 100^{a}	2097 ± 88^{ab}	1864 ± 92^{b}
	Lysine	14 ± 1^{a}	13 ± 2^{a}	11 ± 1^{ab}	7 ± 1^{b}	39 ± 3	38 ± 3	36 ± 2	38 ± 4
	Arginine	41 ± 6	44 ± 10	34 ± 6	22 ± 2	757 ± 43	780 ± 46	755 ± 25	729 ± 45
	Asparagine	370 ± 87	366 ± 114	219 ± 53	219 ± 46	202 ± 13^{a}	206 ± 6^{a}	177 ± 11^{ab}	164 ± 5^{b}
	Alanine	83 ± 19	66 ± 10	51 ± 8	43 ± 17				
	Threonine	140 ± 14^{a}	110 ± 28^{ab}	86 ± 11^{ab}	73 ± 14^{b}	59 ± 2^{a}	56 ± 3^{a}	53 ± 2^{ab}	47 ± 2^{b}
	Glutamic acid	178 ± 40	176 ± 66	87 ± 15	66 ± 10	336 ± 17^{a}	321 ± 20^{a}	293 ± 16^{ab}	264 ± 19^{b}
	Proline	325 ± 23^{ab}	353 ± 63^{a}	163 ± 58^{c}	198 ± 34^{bc}	2876 ± 67^{a}	2882 ± 79^{a}	2663 ± 115^{ab}	2583 ± 51^{b}
	Valine	54 ± 5^{a}	47 ± 8^{a}	45 ± 3^{a}	27 ± 2^{b}	346 ± 26	324 ± 20	323 ± 28	308 ± 30
	Methionine	6 ± 0^{a}	-	5 ± 0^{ab}	4 ± 0^{b}	30 ± 2	30 ± 1	30 ± 1	27 ± 1
	Isoleucine	75 ± 4^{a}	59 ± 11^{ab}	65 ± 6^{a}	42 ± 5^{b}	192 ± 20	188 ± 17	194 ± 20	176 ± 21
	Leucine	79 ± 2^{a}	64 ± 10^{a}	70 ± 6^{a}	41 ± 6^{b}	159 ± 14	152 ± 13	160 ± 13	149 ± 14
	Tyrosine	30 ± 3^{a}	28 ± 5^{a}	25 ± 2^{ab}	17 ± 2^{b}	168 ± 21	157 ± 16	161 ± 16	159 ± 22
	Phenylalanine	42 ± 5^{a}	42 ± 9^{a}	42 ± 5^{a}	23 ± 1^{b}	427 ± 71	356 ± 40	380 ± 50	388 ± 66
	Tryptophan	32 ± 3^{a}	31 ± 6^{a}	30 ± 3^{a}	18 ± 1^{b}	139 ± 7	179 ± 27	194 ± 31	179 ± 35
	Histidine	14 ± 1	15 ± 5	12 ± 1	8 ± 1	-	-	-	-
	Aminobutyric Acid	1120 ± 97^{a}	1170 ± 141^{a}	995 ± 164^{a}	538 ± 38^{b}	-	-	-	-

Table 4 Average values (mean ± SE) of the identified amino acids after protein hydrolysis of leaves samples from control plants (CT), plants treated with auxin (IAA), with HS from IHSS (IHSS), and with HS from earthworm faeces (EF). Values are expressed in ug/mg $^{-1}$ of dry matter. Letters indicate significant differences among treatments ($P \leq 0.05$) based on Duncan post hoc tests.

Aminoacids	Leaves			
	CT	IAA	IHSS	EF
Lysine	4.88 ± 0.40	4.91 ± 0.25	5.63 ± 0.18	5.43 ± 0.34
Arginine	7.80 ± 0.45^{b}	8.51 ± 0.24^{b}	10.00 ± 0.30^{a}	9.66 ± 0.38^{a}
Glycine	11.75 ± 0.73^{ab}	12.08 ± 0.48^{a}	10.25 ± 0.43^{bc}	9.77 ± 0.42^{c}
Serine	15.91 ± 0.86^{a}	13.11 ± 1.01^{b}	10.81 ± 0.59^{bc}	10.66 ± 0.67^{c}
Alanine	9.25 ± 0.90^{a}	7.23 ± 0.35^{b}	6.46 ± 0.25^{b}	5.92 ± 0.30^{b}
Aspartic acid	22.64 ± 1.56^{b}	24.46 ± 1.53^{b}	28.95 ± 1.30^{a}	25.92 ± 1.6^{ab}
Threonine	0.84 ± 0.35	0.85 ± 0.20	0.91 ± 0.24	1.03 ± 0.32
Glutamic acid	50.62 ± 3.98^{a}	40.72 ± 3.36^{b}	38.97 ± 2.30^{b}	34.99 ± 0.80^{b}
Proline	10.19 ± 0.39^{b}	11.59 ± 0.41^{ab}	13.22 ± 0.75^{a}	12.88 ± 0.55^{a}
Valine	5.31 ± 0.49	5.70± 0.33	6.23 ± 0.41	6.68 ± 0.44
Isoleucine	3.68 ± 0.41	3.97 ± 0.27	4.38 ± 0.38	4.76 ± 0.36
Leucine	13.81 ± 0.81^{b}	15.62 ± 0.29^{ab}	15.76 ± 0.82^{ab}	16.2 ± 0.78^{a}
Phenylalanine	7.84 ± 0.52^{b}	8.87 ± 0.21^{ab}	9.84 ± 0.32^{a}	10.06 ± 0.52^{a}

Canonical discriminant analysis

CDA was used to evaluate the influence of the treatments on the studied parameters. It was not possible to perform CDA analysis on roots data as the number of observations was not enough to generate a model.

The scatter plot of MCW extracted metabolites is reported in Fig. 3. The CDA model explained 93.3 % of the variability (Wilk's Lambda value = 0.003, F = 2.79, $P \leq 0.001$); particularly Can 1 explained 68.5 %, well distinguishing between CT and IAA vs. IHSS and EF, whereas Can 2 explained 24.8 %, discriminating IHSS from EF. Analyzing the Malahanobis quadratic distances of the four clouds (Table S1 of the supplementary material), it was possible to determine that IHSS and EF were significantly different from CT ($P \leq 0.01$ and $P \leq 0.05$ respectively) and from IAA ($P \leq 0.01$ and $P \leq 0.05$ respectively), and EF from IHSS ($P \leq 0.05$). Sucrose ($P \leq 0.001$), glutamine ($P \leq 0.05$), threonine ($P \leq 0.01$), and glutamic acid ($P \leq 0.05$) significantly contributed to the CDA model (Table S2). Moreover, sucrose ($P \leq 0.001$), asparagine ($P \leq 0.05$), threonine ($P \leq 0.05$), and glutamic acid ($P \leq 0.05$), correlate well with Can1, meanwhile sucrose ($P \leq 0.05$), glutamine ($P \leq 0.05$), and threonine ($P \leq 0.01$) with Can 2 (Table S2).

In Fig. 4 the scatter plot of amino acids from proteins hydrolysis is reported. The model well distinguished IHSS and EF from CT ($P \leq 0.001$), and from IAA ($P \leq 0.05$) (Table S3) (95% of the variability interpreted, Wilk's Lambda value = 0.005, F = 2.21, $P \leq 0.01$); Can 1 explained 87.4% of the model variability, and Can 2 9.5%. Arginine, glycine, serine, alanine, proline, phenylalanine, and glutamic acid significantly contributed to construction of CDA model (Table S4); the above amino acids plus aspartic acid, valine, isoleucine, and leucine significantly correlated with Can 1, and only glycine with Can 2 (Table S4).

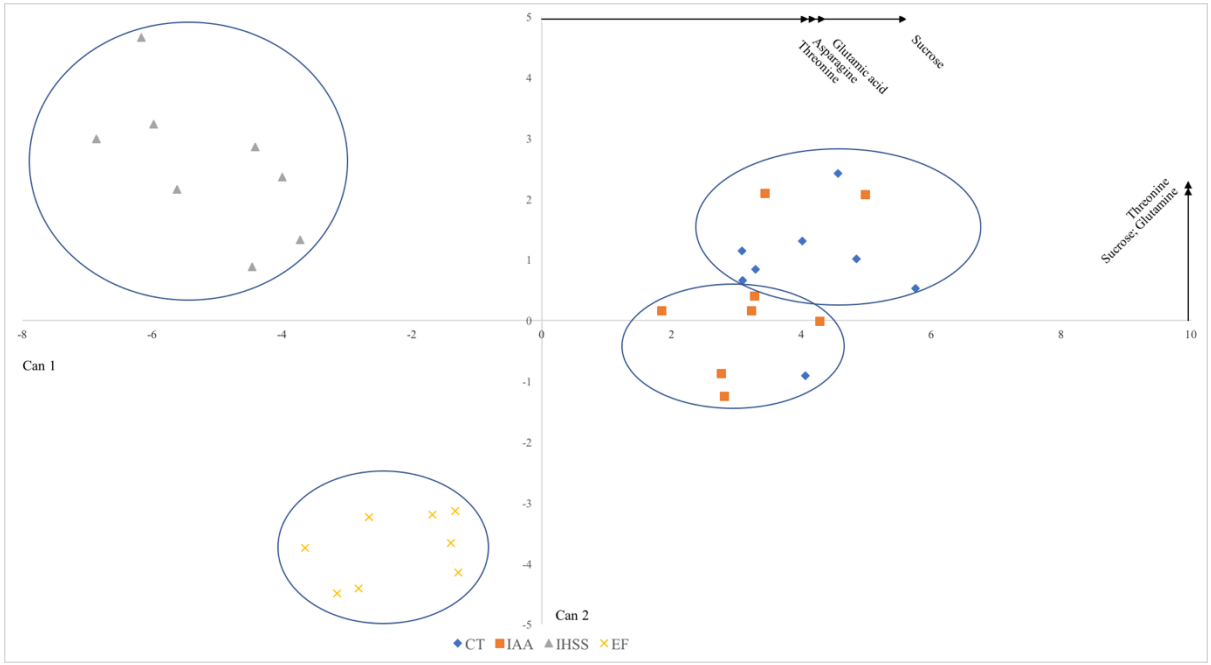

Fig. 3 Scatter plot of CDA of carbohydrates, sugar alcohol, and free amino acids. The separation of the samples is based on the treatment condition. Additional axes show the most important variables contributing to the formation of the canonical variables (Can 1 explained 68.5 %, whereas Can 2 explained 24.8 %). The extension of each arrow from the plot center indicates the importance of the variable in the canonical structure according to Pearson correlation coefficients and its direction reports the increase or decrease of the variable.
CT = control; IAA = auxin; IHSS = humic substances from International humic substances society; EF = humic substances from earthworm *faeces*

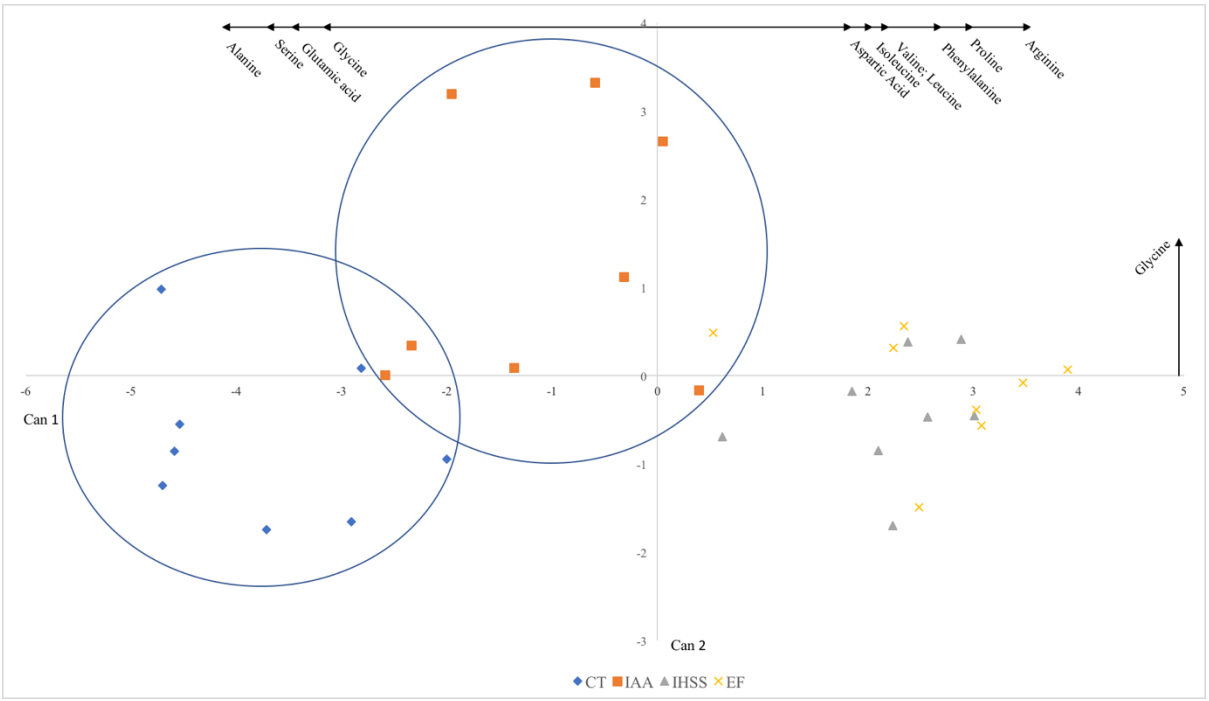

Fig. 4 Scatter plot of CDA of amino acids derived from the protein hydrolysis. The separation of the samples is based on the treatment condition. Additional axes show the most important variables contributing to the formation of the canonical variables (Can 1 explained 87.4% of the model variability, and Can 2 9.5%). The extension of each arrow from the plot center indicates the importance of the variable in the canonical structure according to Pearson correlation coefficients and its direction reports the increase or decrease of the variable.
CT = control; IAA = auxin; IHSS = humic substances from International humic substances society; EF = humic substances from earthworm *faeces*

Discussion

It is well documented that HS increase plant growth, root system development (Canellas et al. 2002; Trevisan et al. 2010; Zandonadi et al. 2007), and several physiological processes (Conselvan et al. 2017; Nardi et al. 2009). In growing cells/organs there is a higher production of proteins, demand of amino acids, and over-working of the respiratory pathway to support the elevated active protein synthesis and energy request (Fernie et al. 2004; Hildebrandt et al. 2015).

Eight hours of treatment with HS from earthworm *faeces* induced a significant reduction of the concentration of all detected carbohydrates and most of the free amino acids in roots, compared to the control, probably related to an overall increase in plant metabolism. From IR, NMR spectra and CDA analysis of sugars and amino acids we could observe that HS with different chemical structure (IHSS and EF) caused different physiological answers in treated plants.

HS influence on carbohydrate concentration

Arabidopsis plants treated with EF had a significant reduction of sucrose, fructose, glucose, and mannose content in roots, and sucrose in leaves (Table 3). It is known that HS have an influence on carbohydrate metabolism, affecting the level and distribution of sugars in treated plants. Canellas et al. (2013) reported that maize plants treated with HS had a lower leaf content of free carbohydrates with a reduction by 60 % of glucose, fructose, and starch compared to the control. Nardi et al. (2007) demonstrated that on maize plant the Fraction III of HS and humic acids (HA) extracted from Fulvudand soil positively influenced the activity of glycolysis (glucokinase, phosphoglucose isomerase, PPi-dependent phosphofructokinase and pyruvate kinase) and TCA cycle enzymes (citrate synthase and malate dehydrogenase).

Sucrose is synthetized in leaves and it is the major end product of photosynthesis and transport sugar in plants. It is a source of energy and carbon for organic and storage compounds (Rolland et al. 2006; Winter and Huber 2000) as well as the starting point for the respiratory pathway, being the principal substrate for glycolysis. A lower concentration of sucrose in roots and leaves could be due to a higher activity of glycolysis to support metabolic processes stimulated by HS leading to enhanced plant growth. Such a response was also hypothesized by Canellas et al. (2013) as an effect of HS and *Aspergillus* treatment. This mechanism is also consistent with

the reduction of glucose concentration in roots and leaves, and fructose in roots. Although in roots of EF treated plants all carbohydrates were significantly lower in concentration, leaf sugars concentration might be replenished by higher photosynthesis, which has been previously reported as an effect of HS treatment (Aguiar et al. 2016).

HS influence on amino acid concentration

Free amino acids are the building blocks of proteins, but they are also involved in numerous cellular reactions and they influence different physiological processes as plant growth and development, resistance to biotic and abiotic stress, production of metabolic energy (Galili et al. 2014; Hildebrandt et al. 2015; Pratelli and Pilot 2014). Free amino acids could be catalyzed through deamination reaction: nitrogen is removed as ammonium from the amino acids and transferred to storage compounds. If required, ammonium can be re-assimilated by glutamine synthase and used to produce new amino acids. The remaining carbon skeletons of catalyzed amino acids are commonly converted to precursors or intermediate of tricarboxylic acid (TCA) cycle (Hildebrandt et al. 2015).

The lower concentration of free amino acids in EF treated plants might may be due to the higher protein production to support enhanced plant growth. This hypothesis is confirmed by the higher concentrations of leucine, proline, arginine, and phenylalanine obtained with protein hydrolysis extraction (Table 4). However, it cannot be ruled out that free amino acids are depleted for energy purposes. Finally, free amino acids contents might not be restored to control levels due to the short treatment duration of eight hours.

Amino acids could also be precursors for the synthesis of several secondary metabolites (e.g. phenylpropanoids, alkaloids, glucosinolates) involved in some plant functions such as signaling (e.g. hormones), structure (e.g. lignin), defense (e.g. glucosinolates), and protection (e.g. pigments) (D'Auria and Gershenzon 2005). In particular the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) are the precursors for the biosynthesis of numerous natural products, such as pigments, alkaloids, hormones, and cell wall components (Maeda and Dudareva 2012). In EF treated roots the lower concentration of phenylalanine among free amino acids could be related to higher production of lignin (Rennie and Scheller 2014), or phenolic compounds with signaling function (Murphy et al. 2000; Suzuki et al. 2004).

Increased phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity with consequent phenylalanine degradation is a known effect of HS extracted from various sources (Conselvan et al. 2017). In roots of plants treated with EF was also detected a lower tryptophan concentration with respect to untreated ones. The importance of this amino acid is related to the auxin indole-3-acetic acid biosynthesis (IAA) (Mano and Nemoto 2012). Auxins are directly involved in the plant growth and development (Quint and Gray 2006). In particular, IAA is responsible for root system architecture and different stages of root development (Overvoorde et al. 2010), with a pivotal role in primary root, lateral root, and root hair development (Benfey et al. 2010). The hypothesis that, in EF treated plants, free tryptophan is consumed to produce IAA is supported by Trevisan et al. (2010) where they found that HS have an influence on the regulation of auxin-inducible genes. However, in our study, IAA treatment did not have the same activity on tryptophan concentration like EF. These observations could suggest that, even if HS are known to have an auxin-like activity, they also have a direct influence on the auxin metabolic pathway, which could only in part explained with the presence of IAA in the HS matrix.

CDA analysis

CDA analysis successfully distinguished the different treatments (Fig. 3 and 4, Tables S1 and S3). HS, both EF and IHSS, were significantly different from CT and IAA, although CT and IAA scatters were not discriminated by the model. This suggests that IAA treatment, with the concentration found in HS (Trevisan et al. 2010), was not able to induce the same responses elicited by HS. Although the auxin-like effect of HS has been debated at length (Nardi et al. 2002), this results confirms the hypothesis that the activity of HS is not only due to the IAA present in HS molecules, but also because of other compounds with a similar activity (Nardi et al. 2009). HS may behave as a signal of the rhizosphere, perhaps eliciting phytohormone production at a plant and/or at a soil biota level. Moreover, CDA analysis of the MCW extracted compounds (Fig. 3) highlighted a clear distinction between EF and IHSS scatter plots. This result accounts for HS compositional differences. Rose et al. (2014) reported that plant responses to HS are not always the same, but they depend on the source and structure of HS, plant type, and environment conditions. The responses elicited by HS have been found to depend on their structure (Canellas et al. 2012): diversely structured humic extracts may cause the different plant physiological responses. In our case study, IHSS and EF were characterized

by different functional groups and structures. IHSS showed a slightly higher aromatic and aliphatic features (hydrophobic fraction of HS) with respect to EF. Aguiar et al. (2013); Canellas et al. (2010); Martinez-Balmori et al. (2014) demonstrated that aliphatic and aromatic components are capable to induce lateral root emergence and stimulate plant growth. The hydrophobic humic components are able to incorporate bioactive molecules into HS structure and protect them against degradation (Piccolo 1996). The organic acids present in plant roots exudates can disaggregate HS into smaller humic molecules (Piccolo and Spiteller 2003). The released bio-active molecules can then access cell membranes and induce physiological responses. The lower content of hydrophobic compounds in EF could facilitate the disaggregation of HS by the organic acids present in root exudates, thus making more available biological active molecules entrapped in HS structure.

Moreover, the higher content of carbohydrates and peptide components (hydrophilic fraction of HS) in EF could explain the higher induction of plant physiological responses. Piccolo et al. (1992) and Nardi et al. (2007) demonstrated that low molecular fraction of humic acids, characterized by high content of hydrophilic compounds, significantly increased nitrogen uptake, glycolytic pathway and Krebs cycle in maize seedlings.

In conclusion, with the present study, we confirm that plants under HS treatments enhance protein and energetic metabolism to support/uphold a higher growth rate and we corroborate that HS extracted from different sources do not elicit the same plant responses. In particular, the relative abundances of aliphatic and aromatic compounds and the presence of carbohydrates and peptide components, as revealed by spectrometric analyses, might be responsible for the different effects of the HS. Auxin-like activity have been previously advocated to explain responses to HS. Our results demonstrate differences between plants treated with IAA and HS, highlighting that responses in terms of carbohydrates and amino acid concentrations can only in part be ascribed to the effects of IAA entrapped in the HS matrix. The presence of other molecules entrapped in the HS matrix could be advocated to justify their biostimulant effects.

Present results can contribute to identifying HS-responsive metabolites, also in relation to the substances structural characteristics, which can be chosen as markers of these compounds bioactivity.

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Supplementary material

Table S1 CDA cloud Mahlanobis quadratic distances with significance considering all the treatments (CT, IAA, IHSS, and EF) for data of the metabolites from the MCW extraction.
Asterisks indicate p values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Treatment	CT	IAA	IHSS	EF
CT	0.0	12.2	90.7**	63.4*
IAA		0.0	80.0**	51.9*
IHSS			0.0	48.3*
EF				0.0

Table S2 Univariate statistics and Pearson coefficients for CDA model considering data of metabolites derived from MCW extraction. Can 1 and Can 2 relate the position of each variable within the first and second canonical axis respectively. Asterisks indicate p values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Metabolite	Univariate F test	Pearson Correlation	
		Can 1	Can 2
Fructose	1.62	0.209	0.180
Glucose 1	0.55	0.124	0.145
Glucose 2	0.53	0.124	0.142
Sucrose	8.20***	0.562***	0.436*
Mannose	0.80	-0.126	0.133
Myo-inositol	2.50	0.320	0.291
Glutamine	3.35*	0.317	0.435*
Lysine	0.23	0.132	-0.077
Arginine	0.27	0.091	0.117
Aparagine	2.33	0.423*	0.184
Threonine	4.95**	0.410*	0.458**
Glutamic acid	3.78*	0.437*	0.315
Proline	1.35	0.177	0.314
Valine	0.43	0.177	0.129
Methionine	0.53	0.103	0.224
Isoleucine	0.27	-0.017	0.178
Leucine	0.15	-0.011	0.118
Tyrosine	0.06	0.025	0.034
Phenylalanine	0.08	0.039	0.057
Tryptophan	0.03	-0.040	0.046

Table S3 CDA clouds Mahlanobis quadratic distances with significance considering all the treatments (CT, IAA, IHSS, and EF) for amino acids derived from proteins hydrolysis.
Asterisks indicate p values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Treatment	CT	IAA	IHSS	EF
CT	0.0	11.4	36.4***	41.5***
IAA		0.0	14.5*	16.9*
IHSS			0.0	3.0
EF				0.0

Table S4 Univariate statistics and Pearson coefficients for CDA model considering data of the amino acids derived from proteins hydrolysis. Can 1 and Can 2 relate the position of each variable within the first and second canonical axis respectively. Asterisks indicate p values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Amino acid	Univariate F test	Perason Correlation	
		Can 1	Can 2
Lysine	0.79	0.282	-0.132
Arginine	8.41***	0.715***	-0.113
Glycine	4.52*	-0.531***	0.393*
Serine	4.93**	-0.625***	0.002
Alanine	6.89**	-0.692***	-0.035
Aspartic acid	1.61	0.379*	-0.020
Threonine	0.09	0.086	-0.025
Proline	5.25**	0.309***	0.205
Valine	2.26	0.441*	-0.152
Isoleucine	1.98	0.414*	-0.147
Leucine	1.91	0.434*	0.026
Phenylalanine	3.57*	0.540**	-0.033
Glutamic acid	4.67**	-0.588***	-0.247

5 Manuscript III

Biostimulant activity of humic substances extracted from leonardites

Giovanni Battista Conselvan[a], Diego Pizzeghello[a]*, Ornella Francioso[b], Michele Di Foggia[c], Serenella Nardi[a], Paolo Carletti[a]

[a] Dipartimento di Agronomia, Animali, Alimenti, Risorse Naturali e Ambiente (DAFNAE), Università di Padova, Viale dell'Università 16, 35020 Legnaro (Padova), Italy
[b] Dipartimento di Scienze Agrarie, Università di Bologna, Viale Fanin 44, 40127 Bologna, Italy
[c] Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Via Belmeloro 8/2, 40126 Bologna, Italy
*Corresponding author: diego.pizzeghello@unipd.it; Tel.: +39-049-827-2915

Plant and Soil

Abstract

Background and aims Biostimulants are natural compounds that enhance plant growth and plant nutrient use efficiency. In this study, biostimulant effects of humic substances (HS) extracted from leonardites were analysed on the metabolism of maize plants grown in hydroponic conditions.

Methods HS extracted from four leonardites were tested for their auxin-like and gibberellin-like activities. Then, 11 day old maize seedlings were treated for 48 h with five concentrations (0, 0.1, 0.5, 1, and 10 mg C L^{-1}) of HS. After sampling, root growth and morphology, glutamine synthetase (GS) activity, glutamate synthase (GOGAT) activity, total protein content, soluble sugars content, phenylalanine ammonia-lyase (PAL) activity, soluble phenols, and free phenolic acids were analysed.

Results HS from leonardites had similar spectroscopic pattern, with small differences. The HS from the South Dakota lignite (HS_USA) had more carboxylic groups, whereas the three from Turkish mines had more aromatic and aliphatic structures. HS_USA best enhanced total root growth, root surface area, and proliferation of secondary roots. Plant nutrient use efficiency was enhanced by HS_4, HS_USA and HS_B, with increment of GS and GOGAT enzymes activity and total protein production. HS stimulated also PAL enzyme activity, followed by a higher production of total soluble phenols, *p*-hydroxybenzoic acid, *p*-coumarilic acid, and chlorogenic acid.

Conclusion This study found that, although the activity of the HS depended on the origin of the leonardite, these compounds can be attributed to the biostimulant products, eliciting plant growth, nitrogen metabolism, and accumulation of phenolic substances.

Keywords

Humic substances, leonardite, biostimulant, glutamine synthetase, glutamate synthase, phenylalanine ammonia-lyase, FT-IR.

Introduction

The development of sustainable and environmentally friendly agricultural systems represents a major policy challenge in many countries (Povero et al. 2016). In the last two decades, farming expectations have changed and farmers are expected to produce food, whilst protecting biodiversity, soil, air and water quality (OECD 2013). Although fertilizers are powerful tools for increasing yield and plant health (Hirel et al. 2001), farmers must optimize product application to avoid nutrient pollution and to preserve the economic margin.

One of the most promising solutions to achieve these goals is the use of plant biostimulants. In 2012 Europe has become a leading market for biostimulants, while the biostimulants global market is expected to grow over the next years, reaching $2,524.02 million in sales by 2019, with an annual growth rate of 12.5% (Calvo et al. 2014; Povero et al. 2016).

Biostimulants are compounds containing substances and microorganisms able to enhance plant nutrient uptake, nutrient efficiency, tolerance to abiotic/biotic stresses, and crop quality (European Biostimulant Industry Council 2013). Furthermore, when applied to the soil, biostimulants may stimulate rhizosphere microbes and soil enzymes, the photosynthetic process, and the production of hormones or growth regulators in plants (Calvo et al. 2014).

Biostimulants are considered as borderline substances between plant protection products and fertilizers, as they do not give direct protection against pest and do not have nutritional activity (La Torre et al. 2016). For this reason, there is not yet a legal definition of biostimulants (du Jardin 2015). Despite this, international organizations and scientists recognized six main categories: microorganisms, protein hydrolysates, seaweed extracts, chitosan, inorganic compounds and humic substances (HS) (Calvo et al. 2014; du Jardin 2015; European Biostimulant Industry Council 2016).

Among these categories, HS or humates have a positive effect on the uptake of macro and micro nutrients that considerably improve the metabolism, the growth and yields of relevant agricultural crops (Bronick and Lal 2005; Ferreras et al. 2006; Nardi et al. 2009; Puglisi et al. 2009). The positive effects of HS on plant metabolism are well recognized as hormone-like activity (auxin, gibberellin or cytokine-like activity) in terms of changes in root architecture through the lateral roots and root hair production (Canellas et al. 2011; Mora et al. 2012; Pizzeghello et al. 2013; Trevisan et al. 2010b). HS increase root plasma membrane H^+-ATPase activity, enhancing nitrate and other nutrient uptake, contributing to cell wall loosening, cell

enlargement and organ growth (Jindo et al. 2012; Zandonadi et al. 2007). Moreover, TCA cycle, phenylpropanoid metabolism, and uptake and metabolism of nitrate have been found to be positively influenced by treatment with HS (Quaggiotti et al. 2004; Vaccaro et al. 2009).

However, HS effects on plant growth cannot be overgeneralized due to their different origin (e.g. from volcanic soil, compost, vermicompost or brown coal), dosage (differs from types culture media) as well as plant species (Nardi et al. 2009; Rose et al. 2014).

Leonardite is an oxidized form of lignite with a medium-brown coal-like appearance. It is found at shallow depth over more compact coal in various coal mines (Stevenson 1979) around the world, mainly in the USA (Fernandez et al. 1996). This brown coal, particularly enriched in humic C (30-80%), is used to manufacture a wide range of commercial HS products.

Akinremi et al. (2000) demonstrated that leonardite increased dry matter yield and nutrient uptake (N, P, K, and S) when applied to canola. In greenhouse conditions, HS from leonardites enhanced the resistance of tomato plants under salinity stress (Casierra-Posada et al. 2009). *Arnica montana* L. treated with HS from leonardite had higher floral stems' number, flower heads' number, and yield compared to control plants (Sugier et al. 2013). A low molecular weight fraction of HS from leonardite enhanced the seedling's, root surface area, root length, and total root number of snap bean (Qian et al. 2015). David et al. (2014) demonstrated that potassium humate salts extracted from lignite, and potassium humate regenerated from lignite with two oxidizing agents (nitric acid and hydrogen peroxide), positively influenced root growth and division, starch and protein contents in treated *Zea mays* seedlings.

Leonardite is thus referred to as a benchmark humic material with respect to responses on plant growth. Although the effects of leonardite on crop production, resistance to stress, and soil microbial activity have already been reported, much less attention has been devoted to their impact on plant physiology and biochemistry (Bulgari et al. 2015). Moreover, comparison of leonardite from different sources and the growth effects of these differently sourced materials are scarse in the literature. This study seeks to gain a better understanding of the importance of leonardite origin on their biological activity.

HS extracted from four leonardites were characterized by FT-IR. The effects of these HS on *Zea mays* plants grown under controlled conditions were studied by evaluating: (1) roots growth parameters like total root length, area, diameter, thin roots length, and number of tips and forks; (2) the responses of enzymes involved in nitrogen and phenylpropanoid metabolism, (3) proteins, sugars, and total phenols content in roots and leaves.

Materials and Methods

Leonardites origin and humic substances extraction

Leonardites have been supplied by LandLab srl (Quinto Vicentino, Vicenza, Italy). The leonardite named LE_USA are from South Dakota mines, while the others, LETU_4, LE_A, and LE_B are from Turkish mines.

Humic substances were extracted from the four leonardites (LE_USA; LETU_4; LE_A; LE_B) with 0.1M KOH (1:10 w/v) at 130 rpm for 16 h at 50 °C. The extracts were centrifuged at 7000 rpm for 30 min, and filtered on Whatman filter N. 2 paper (Whatman, Boston, USA). Humic extract was desalted by using 14 kDa cut-off dialysis Visking (Medicell, London, UK) tubing with distilled water. Distilled water was changed daily until neutral pH was reached. Subsequently, the extracts were desalted on ion exchange Amberlite IR-120 (H^+ form) (Stevenson 1994). 20 ml of humic extracts were freeze-dried for IR, CNS, and ash content determination and the remaining extracts were kept frozen for use on plant treatments.

Humic carbon content of the extracts in each step of the extraction was determined in triplicate by following a modified version of the method of Walkley and Black (1934). Humic substances (HS) were labelled as HS_USA, HS_4, HS_A, and HS_B.

Chemical and FTIR characterization

The ash content of leonardites and humic substances was determined gravimetrically after dry combustion in a muffle furnace at 550 °C for 6 h. The moisture content was determined at 105 °C. The pH was measured potentiometrically on pulverized samples by adding deionized water (1:2.5 w/v, dry weight basis). The electrical conductivity (EC) for leonardites only, was potentiometrically determined after water extraction (2:5 w/v) and filtration through Whatman filter N. 2 paper.

Total C, N, and S contents were measured in triplicate on each sample by using CNS (Carbon, Nitrogen and Sulphur) Vario Macro elemental analyzer (Elementar, Hanau, Germany).

IR spectra were recorded with a Bruker ALFA FT-IR Spectrophotometer (Bruker, Ettlingen, Germany) equipped with a single reflection ATR sampling module. The spectra were collected from 4000 to 400 cm^{-1} and averaged over 64 scans (resolution 4 cm^{-1}). The spectral data were processed with Grams/386 spectroscopic software (Galactic Industries, Salem, NH, USA).

Curve-fitting analysis in the region between 1900-900 cm^{-1} was used to determine the area under each of the individual bands by using Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH).

Bioassay to test the biological activity of HS from leonardites

The biological activity of HS_USA, HS_4, HS_A, and HS_B was assessed by checking the growth reduction of watercress (*Lepidum sativum* L.) roots and the increase of the length of lettuce (*Lactuca sativa* L.) shoots (Audus 1972).

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide (Sigma, St. Louis, MO) for 15 minutes. After rinsing 5 times with sterile distilled water, 20 seeds were aseptically placed on filter paper in a Petri dish. For watercress, the filter paper was wetted with 1.2 mL of H_2O (control); or 1.2 mL of 0.1, 1, 10, and 20 mg L^{-1} indole-3-acetic acid (IAA) (Sigma, St. Louis, MO) to obtain the calibration curve; or 1.2 mL of a serial dilution (10 mg C L^{-1}, 1 mg C L^{-1}, 0.1 mg C L^{-1}, 0.001 mg C L^{-1}, and 0.00001 mg C L^{-1}) of the HS. For lettuce, the experimental design was the same as for watercress, except that the sterile filter paper was wetted with 1.4 mL of the above HS (dilutions 10 mg C L^{-1}, 1 mg C L^{-1}, 0.1 mg C L^{-1}, 0.001 mg C L^{-1}, and 0.00001 mg C L^{-1}) and the calibration curve was a serial dilution of 0, 0.01, 0.1, and 10 mg L^{-1} gibberellic acid (GA) (Sigma).

The seeds were germinated in the dark at 25 °C. After 48 h for watercress and 72 h for lettuce, the seedlings were removed and the root or shoot lengths were measured.

A linear regression model (Y = a + bX) was applied to describe the dose-response relationship. In the case of IAA, GA and HS doses a mathematical transformation to log(x) (where x is the original dose value) was needed before regression analysis (Pizzeghello et al. 2013).

Plant material and growth conditions

Plant material was grown as reported in Carletti et al. (2008). Seeds of *Zea mays L.* (var. DKC 5401, DeKalb, Italy) were soaked in distilled water for one night. Seeds were left to germinate on filter paper wetted with 1 mM $CaSO_4$ for 60 h in the dark at 25 °C. Germinated seedlings were transplanted into 3 L beakers containing an aerated Hoagland solution (Hoagland and Arnon 1950) with a density of 24 plants per beaker.

The nutrient solution was renewed every 48 h and had the following composition: 40 μM KH_2PO_4, 200 μM $Ca(NO_3)_2$, 200 μM KNO_3, 200 μM $MgSO_4$,10 μM FeNaEDTA, 4.68 μM

H_3BO_3, 0.036 µM $CuCl_2 \cdot 2H_2O$, 0.9 µM $MnCl_2 \cdot 4H_2O$, 0.086 µM $ZnCl_2$, 0.011 µM $NaMoO \cdot 2H_2O$.

Plants were grown in a climate chamber with 11 h of light per day, air temperature between 21 and 27 °C, relative humidity of 70/85%, photon flux density of 280 mol m^{-2} s^{-1}. Nine days after transplanting, HS_USA, HS_4, HS_A, and HS_B were added to the nutrient solution contained in the beakers at different concentrations: 0 (control), 0.1, 0.5, 1 and 10 mg C L^{-1}. Each concentration was replicated 3 times. The addition of the products to the nutrient solution was performed only once. After 48 h, plants were randomly harvested, fresh samples of roots and leaves were carefully washed and dried with blotting paper, and weighted (data not shown). The treatment period was chosen according to previous experience on studies of HS from various origins and their related biostimulant activity (Ertani et al. 2011; Quaggiotti et al. 2004).

A subsample of the plant material was immediately frozen with liquid nitrogen and kept at -80 °C for physiological analyses. Dry weight measurement was performed in triplicate for each treatment using aliquots of approximately 1 g plant fresh tissue.

For each beaker roots and leaves were weighed. The samples were placed in a drying oven for 2 days at 70 °C and allowed to cool for 2 h inside a closed bell jar. The dry weight was measured per plant (data not shown).

Root scanning

Root scanning was rapidly performed before the sampling process using an Epson Expression 10000XL 1.0 system (Regent Instruments Company, Canada) as reported in Ding et al. (2014). Three plants for each beaker were randomly picked for root scanning, for a total of 9 plants for each measurement. The following parameters were recorded with a root image analysis system using the image analysis software WinRHIZO Pro (Regent Instruments, QC, Canada): root total length (TRL) (cm), surface area (cm^2), average diameter (mm), number of tips, and length of fine roots (cm) ($0 < L < 0.5$).

Protein extraction and determination

Fresh leaf and root samples were ground to a homogenous powder in liquid nitrogen (N_2). Proteins were extracted with 38 mM KH_2PO_4 and 62 mM K_2HPO_4 buffer at a pH 7. The protein concentration in the extract was determined according to Bradford (1976), using a

Jasco V-530 UV/vis spectrophotometer (Jasco Corporation, Tokyo, Japan) at 595 nm. The protein concentration was expressed as mg of protein per g of fresh root or leaf.

Enzyme extraction and essay conditions

To extract the enzymes involved in N reduction and assimilation, fresh leaves and roots were ground to a homogenous powder in liquid N_2. For the extraction of the enzymes two different buffers were used. Each activity essay was done in triplicate.

Glutamine synthetase (GS; EC 6.3.1.2) was extracted by homogenising 0.6 g of ground roots or leaves with 2.4 mL of a 1 mM Tris(hydroxymethyl)aminomethane HCl (Tris-HCl), 25 mM KH_2PO_4, 10 mM L-cysteine hydrochloride monohydrate, 3 % (w/v) bovine serum albumin solution, at 4 °C at pH 7.8 (Baglieri et al. 2014). After 10 minutes, the extract was filtered through three layers of muslin and centrifuged at 15000 *g* for 25 min at 4 °C. 200 µL of supernatant was incubated with 200 µL of reaction buffer (50 mM Tris-HCl, 20 mM $MgSO_4$, 80 mM L-glutamate, 30 mM NH_2OH, 24mM ATP; pH 7.8) at 37 °C for 25 minutes. The reaction was stopped with stopping solution (370 mM $FeCl_2 \cdot 6H_2O$ and 670 mM HCl). Samples were centrifuged at 15000 *g* for 15 minutes. The amount of γ-glutamyl hydroxamate in the supernatant was photometrically (540 nm) determined against an immediately stopped parallel sample (Jezek et al. 2015). A standard curve was made using γ-glutamyl hydroxamate (GHA) (Sigma). The enzyme activity was expressed as µmol of GHA produced per g of fresh root or leaf per minute.

Glutamate synthase (GOGAT; EC 1.4.7.1) was extracted by homogenizing 0.5 g of ground roots or leaves with 2 mL of a 100 mM Tris-HCl at pH 8.2, 10 mM $MgCl_2 \cdot 6H_2O$, 2 mM β-mercaptoethanol, 10 % (v/v) glycerol and 1 mM Na_2EDTA solution. After 15 minutes, the extract was filtered through two layers of muslin and centrifuged at 15000 *g* for 30 minutes at 4 °C. The supernatant was centrifuged a second time at 15000 *g* for 15 minutes at 4 °C. For the enzymatic essay, 100 µL of extract were added to 900 µL of reaction buffer (41.6 mM HEPES at pH 7.5, 1 mM NADH, 10 mM EDTA, 20 mM glutamine) and 300 µL (for leaf extract) or 900 µL (for root extract) of 10 mM α-ketoglutaric acid. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm according to Avila et al. (1987). GOGAT activity was expressed as nmol NADH reduced per g of fresh root or leaf per minute.

For the phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) essay, 1 g of ground leaves were

homogenized with 0.1 g of poly(vinylpolypyrrolidone) (PVPP) and 5 mL of 100 mM potassium phosphate buffer (pH 8.0) containing 1.4 mM β-mercaptoethanol. After 10 minutes, the extract was filtered through two layers of muslin and centrifuged at 15000 *g* for 20 minutes at 4 °C. 60 µL of supernatant was incubated with 400 µL of 100 mM Tris-HCl buffer (pH 8.8), 140 µL of 100 mM phosphate buffer and 200 µL of 40 mM phenylalanine at 37 °C for 30 minutes. The reaction was stopped with 200 µL 6 M HCl (El-Shora 2002). After centrifuging at 10000 g for 15 minutes, the absorbance of the supernatant was measured at 280 nm against an immediately stopped parallel sample. A standard curve was made using cinnamic acid (Sigma). PAL activity was expressed as nmol cinnamic acid produced per mg protein per minute.

Extraction and measurement of soluble phenols

Soluble phenolic acids were extracted by homogenizing 200 mg of crushed leaves with 600 mL of pure methanol. The extract was maintained in ice for 30 minutes and centrifuged at 15000 *g* for 30 minutes at 4 °C. Total phenols were measured according to Arnaldos et al. (2001). 1 mL of 2% Na_2CO_3 and 75 µL of Folin-Ciocalteau reagent (Sigma-Aldrich) were added to 50 µL of phenolic extract. After 15 minutes of incubation at 25 °C in the dark, the absorbance was measured at 725 nm. A standard curve was made by using gallic acid (Sigma). The soluble phenols content was expressed as mg of gallic acid equivalent (GAE) per g of fresh leaf.

Quantitative determination of free phenolic acids by HPLC

Leaves (5 g) were homogenized in methanol (20 mL) with an Ultra Turrax T25 dispenser at 13500 rpm for 30 seconds until uniform consistency. Samples were filtered the first time through a filter paper (589 Schleicher) and a then through cellulose acetate syringe filters (0.45 mm). The extract was then ready for HPLC analysis.

The liquid chromatography system was a Jasco X-LC system consisting of a PU-2080 pump, a MD-2015 multi-wavelength detector, a AS-2055 autosampler, and a CO-2060 column oven. The separation of phenolic acids was carried out on a Tracer Extrasil ODS2 column (5 µm, 250 x 4.6 mm, Teknokroma) operating at 35 °C. The flow rate was set to 1 mL min^{-1}. The mobile phase consisted of water (0.1% formic acid)(A) and methanol (0.1% formic acid) (B). The gradient elution was as follows: 25–70% B over 15 minutes and 70–100% B over 5 minutes to

clean the column. Chlorogenic acid, ferulic acid, *p*-coumaric acid, caffeic acid, and gallic acid were quantified using an HPLC diode array detector (DAD)at 325 nm. Identification of *p*-coumaric acid was performed at 310 nm. ChromNAV chromatography data system was used as software.. All standards were dissolved in methanol and the calibration curves were generated with concentrations ranging from 0.3 mg L^{-1} to 30 mg L^{-1} (Nicoletto et al. 2013).

Quantitative determination of sugars by HPLC

The liquid chromatography used in these analyses was the same reported above. The separation of sugars was achieved on a HyperRez XP Carbohydrate Pb^{++} analytical column (8 μm, 300 x 7.7 mm, ThermoScientific), operating at 80 °C. Isocratic elution was made using water at a flow rate of 0.6 mL min^{-1}. Standards of β-D-glucose, β-D-fructose, and sucrose were dissolved in water and the calibration curves were generated with concentrations ranging from 100 mg L^{-1} to 1000 mg L^{-1} (Nicoletto et al. 2013).

Statistical analysis

The data represent the means of measurements from three different beakers per treatment. For each measurement, the average ± standard error is reported. Analysis of variance (two-way ANOVA) was performed using the SPSS 23 (IBM Corp) software with leonardite type and concentration as factors, and was followed by pairwise post hoc analyses (Student-Newman Keuls test) to determine which means differed significantly at $P \leq 0.05$. Levene and Mauchly's tests were applied to check homoscedasticity and sphericity, respectively, to ensure that assumptions of the model were met as recommended in Field (2013).

For each analytical parameter, linear and logarithmic regressions were also performed using SPSS 23 software with concentrations of HS as independent variable to evaluate the presence of dose-response curves.

Results

Chemical characterization

The main chemical characteristics for each leonardite are displayed in Table 1. The pH ranged from alkaline for LE_USA to acid for LETU_4, LE_A, and LE_B. It is interesting to note that the EC was high in LE_USA and low in LETU_4. LE_USA had high C, N, and S content while LETU_4 had a high mineral content.

Characteristics of HS are shown in Table 2. HS had similar pH values as a consequence of Amberlite IR-120 treatment. HS_4 had the highest C content (50.48%), whereas HS_USA had the highest S content (5.63%).

Table 1 Physical and chemical parameters of leonardites from different origin. Data are mean ± SE, *n*=3

Treatment	$[H^+]$ pH	EC ($\mu S\ cm^{-1}$)	C* ($g\ kg^{-1}$)	N ($g\ kg^{-1}$)	S ($g\ kg^{-1}$)	DW (%)	Moisture (%)	Ash** (%)
LE_USA	8.57±0.05	1400±58	29.81±0.51	3.39±0.15	6.69±0.12	90.00±0.14	10.00±0.14	62.29±0.64
LETU_4	5.29±0.03	128±5	17.84±0.45	0.38±0.02	1.37±0.23	92.55±3.84	7.45±0.86	73.76±1.13
LE_A	3.81±0.05	1002±21	16.04±1.10	0.55±0.04	1.07±0.31	81.00±0.40	19.00±0.40	66.69±0.85
LE_B	3.85±0.04	906±11	17.35±0.64	0.56±0.05	1.02±0.15	81.14±0.37	18.86±0.37	66.94±1.23

EC = Electrical Conductivity
DW = Dry Weight
* = $g\ kg^{-1}$ of DW** = % of DW

Table 2 Elemental analysis and pH of humic substances extracted from leonardites. Data are mean ± SE, *n*=3

Treatment	$[H^+]$ pH*	C** ($g\ kg^{-1}$)	N ($g\ kg^{-1}$)	S ($g\ kg^{-1}$)
HS_USA	2.35±0.01	45.16±0.31	1.06±0.09	5.63±0.52
HS_4	2.64±0.04	50.48±0.15	1.38±0.13	2.86±0.38
HS_A	2.63±0.03	46.94±0.23	1.72±0.27	1.35±0.29
HS_B	2.58±0.01	47.95±0.17	1.76±0.02	1.51±0.41

* = pH measured after amberlite purification of the extract
** = $g\ kg^{-1}$ of DW

FTIR characterization

The main functional groups of HS_USA, HS_4, HS_A, and HS_B are displayed in Fig. 1. In all spectra there are vibrational bands which are assigned to the same functional groups such as the broad band from 4000 to 3000 cm^{-1} arises from (O–H) stretching vibration. The shape of this region suggests that the O-H groups formed several hydrogen bonds. The broad shoulder in the 2700-2400 cm^{-1} region is undoubtedly due to OH stretching of intermolecular hydrogen bonding in carboxylic acids or alcohols (Rao, 1963). The presence of peaks at around 1700 and 1220 cm^{-1} might be due to C=O and C–O stretching motions of carboxylic acids. The peaks appearing at 2910 and 2852 cm^{-1} toghether with those at around 1420 and 1370 cm^{-1} are due to (C-H) stretching and bending motions in aliphatic substances respectively. The strong band at around 1580 cm^{-1} is highly characteristic of aromatic rings skeletal vibration (Bellamy, 1975). Since the position and intensity of this band are dependent on the type of substitution, the conjugation with C=C or C=O should justify the great intensification of this band in all spectra. Other bands that can be useful in identifying the aromatic compounds are the C-H stretching motion at around 3100-3000 cm^{-1} and the C-H out of plane deformation between 900 cm^{-1} and 650 cm^{-1}. The variable intensity of the bands at around 1030 cm^{-1}, 520 cm^{-1} and 463 cm^{-1} may be due to mineral impurities.

Deconvolution fitting procedure on the pattern of HS from different leonardites gave eight Gaussian curve centered at 1700, 1580, 1420, 1210, 1130, 1030, 917 cm^{-1} (Fig. 2). The percentage area for each band considerably changed in relation to different leonardite origin. In particular, the content of COOH (1700 cm^{-1}) linked to aromatic rings (Bellamy, 1975) was higher in HS_USA and progressively decreased in others. In contrast, the aromatic C=C skeletal stretching (1580 cm^{-1}) gradually increased from HS_USA to HS_B. The considerable intensification of this band in HS_A and HS_B might suggest the presence of different polar substituents in the aromatic ring (Bellamy, 1975). As well as the coupled C-O stretching and OH in plane deformation modes (1210 cm^{-1}), which are typical in aryl acids and phenols (Bellamy. 1975), was highest in HS_A and HS_B. At lower frequencies, the C–O and C-C (1130 cm^{-1}) were only present in HS_USA and HS_4, with highest amount in HS_4. The C-O-C stretching in ethers (1030 cm^{-1}) appeared in all samples but the lowest percentage was detected in HS_4. Finally, the coupled C-OH bending out of plane and CH bending (917 cm^{-1}) was considerably higher in USA.

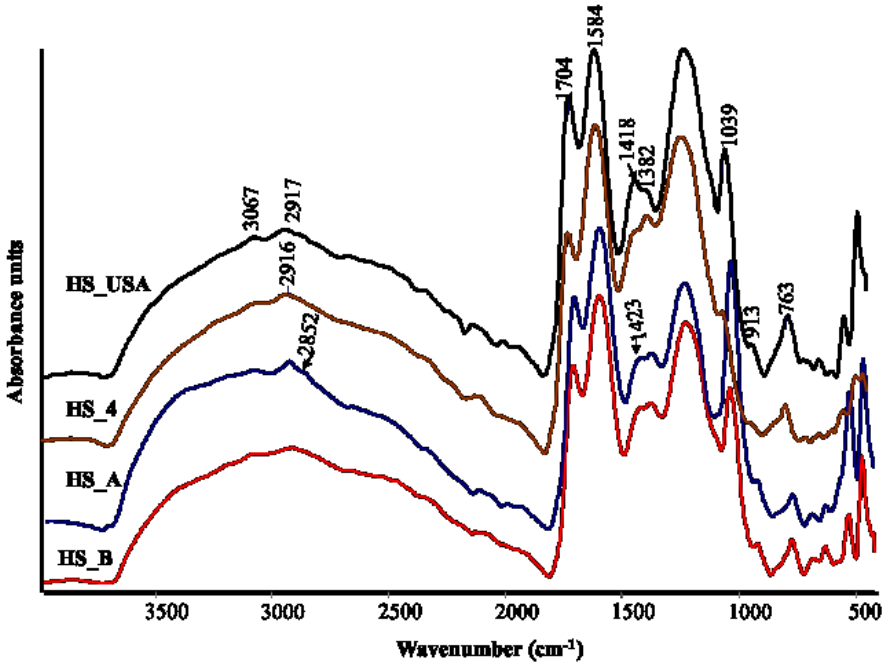

Fig. 1 FT-IR spectra of HS extracted from leonardites (HS_USA, HS_4, HS_A, and HS_B).

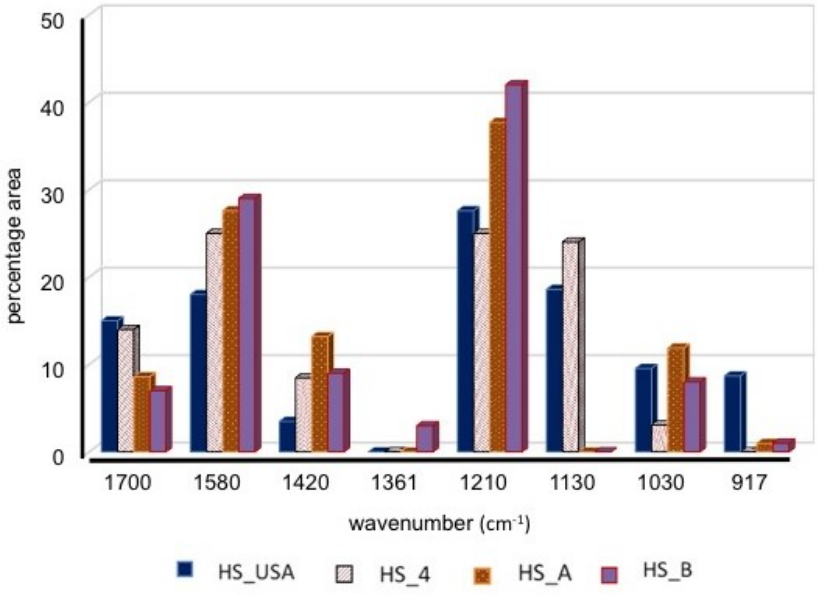

Fig. 2 Histograms of HS of the ATR/FTIR peak areas processed by Gaussian curve fitting.

Bioassay to test the biological activity

IAA concentration in growth media inhibited the elongation of watercress roots in a dose-dependent response ($P \leq 0.001$) (Table 3). In a similar way, the increasing concentrations of HS_USA, USA_4, and HS_A caused significant decreases of watercress roots elongation. In all cases, a logarithmic model explained the best fit of our data (R^2 = 98–99%, $P \leq 0.05$). Only in HS_B there was not a significant dose-dependent response. The b value coefficient was used to compare the auxin-like effect between HS (Table 3). The lower b value was related to the higher auxin-like activity: HS_A had the highest activity, HS_4 the lowest one, while in HS_USA b value was intermediate. For the gibberellin-like activity (Table 3), the dose-dependent response induced by GA was significant ($P \leq 0.05$) in the elongation of lettuce shoots. Also in this experiment, the best fit was obtained with logarithmic dose-response curve (R^2 = 99%) for HS_USA ($P \leq 0.05$) and HS_A ($P \leq 0.05$). The high b value corresponded to a large GA-like activity. Consequently, HS_USA had the highest GA-like activity, and HS_A the lowest one. No GA-like activity was observed for HS_4 and HS_B.

Table 3 Parameters of the regression curves [Y = a + b*log(X)] between concentration and root length of watercress plantlets or steam length of lettuce plantlets treated with indole-3-acetic acid (IAA) or gibberellic acid (GA) or with the humic substances extracted from leonardites

Treatment	R^2	*b*	*P*	df
IAA	0.99	-1.76	0.001	1215
HS_USA	0.99	-0.21	0.040	277
HS_4	0.99	-0.11	0.050	291
HS_A	0.98	-0.46	0.043	316
HS_B	ns	ns	ns	ns
GA	0.93	0.72	0.037	262
HS_USA	0.99	0.29	0.048	249
HS_4	ns	ns	ns	ns
HS_A	0.99	0.07	0.050	247
HS_B	ns	ns	ns	ns

df = degree of freedom;
ns = not significant.

Total root length and other morphological parameters of maize plants

Total radicular length for each seedling was calculated as the sum of the lengths of all radicular nodal segments, using automatic linearization with WinRHIZO software. In maize seedlings treated for two days with HS, root diameter, number of forks, and number of thin roots were

significantly affected by treatment ($P \leq 0.05$) and concentration ($P \leq 0.05$) (Table 4). As a result, a stimulation of total root length (TRL) ($P \leq 0.05$), and enhanced root surface area ($P \leq 0.05$) was obtained. Among HS and with respect to untreated plants, HS_USA affected the greatest number of forks (2305 *vs* 974), number of thin roots (624 *vs* 349), TRL (743 mm *vs* 429 mm), and root surface area (89 cm^2 *vs* 51 cm^2), whereas HS_A had the highest effect on root diameter (0.39 mm *vs* 0.38 mm) and number of tips (477 *vs* 396). The concentrations stimulated in a wider way the aforesaid parameters (Table 4). Indeed, TRL was up to 1.92 fold higher compared to untreated for HS_4 at 0.1 mg C L^{-1} (treatment × concentration interaction significant at $P \leq 0.05$), the number of forks raised up to 2.96 fold compared to untreated for HS_USA at 0.5 mg C L^{-1} (treatment × concentration interaction significant at $P \leq 0.05$), and the number of thin roots were up to 1.99 fold compared to untreated for HS_B at 1 mg C L^{-1} (treatment × concentration interaction significant at $P \leq 0.05$). Finally, although not statistically significant, HS_A at 0.1 mg C L^{-1} gave the highest number in root tips (1.30 fold compared to untreated).

For all the HS linear and logarithmic regression models were tested and they did not explain the data distribution of tested root growth parameters. Parameters (R^2 and P value) are reported in Supplementary material (Table S1).

Table 4 Effect of different concentrations of HS extracted from leonardites on root growth morphological parameters of maize seedlings analysed by WhinRhizo software. Total root length (TRL) is calculated as the sum of the length of primary and lateral roots

Treatment	Dose (mg C L^{-1})	TRL (cm)	Area (cm^2)	Diameter (mm)	Tips (n)	Forks (n)	Thin roots (cm)
Control	0	429±26^{cCc*}	51±3^{cCb}	0.38±0.006^{abcdABab}	396±21	974±78^{deBb}	349±25^{bCd}
HS_USA	0.1	734±12^{abc}	90±16^{abc}	0.38±0.013^{abc}	492±59	2436±555^{ab}	615±101^{ab}
	0.5	757±97^{ab}	97±12^{ab}	0.41±0.009^{ab}	372±41	2880±394^{a}	618±81^{ab}
	1	787±28^{a}	87±3^{abc}	0.35±0.009^{cd}	428±63	2130±98^{abc}	669±36^{a}
	10	695±96^{abc}	79±10^{abc}	0.36±0.007^{abcd}	390±27	1774±347^{bcde}	594±94^{ab}
		743±43^{A}	89±5^{A}	0.379±0.006^{AB}	420±25	2305±199^{A}	624±38^{A}
HS_4	0.1	825±117^{a}	98±12^{a}	0.38±0.008^{abcd}	483±72	2005±372^{bcd}	716±107^{a}
	0.5	505±72^{abc}	57±8^{abc}	0.35±0.007^{abcd}	415±35	1054±148de	429±68^{ab}
	1	606±46^{abc}	65±6^{abc}	0.34±0.007^{cd}	431±23	1321±127^{cde}	530±44^{ab}
	10	594±73^{abc}	67±10^{abc}	0.35±0.015^{bcd}	488±43	1341±286^{cde}	507±64^{ab}
		615±41^{B}	69±5^{B}	0.359±0.005^{C}	451±20	1378±127^{B}	530±37^{B}
HS_A	0.1	613±61^{abc}	76±8^{abc}	0.39±0.007^{abc}	516±45	1401±148^{cde}	494±47^{ab}
	0.5	562±59^{abc}	74±10^{abc}	0.41±0.020^{a}	490±49	1194±188^{cde}	438±41^{ab}
	1	665±35^{abc}	83±6^{abc}	0.39±0.01^{abc}	433±43	1813±120^{bcde}	568±27^{ab}
	10	442±18^{bc}	52±2^{c}	0.37±0.005^{abcd}	458±10	940±43de	354±19^{b}
		562±29^{B}	70±4^{B}	0.394±0.006^{A}	477±19	1299±92^{B}	455±24^{B}
HS_B	0.1	551±44^{abc}	58±5^{abc}	0.33±0.007^{d}	327±27	1139±121^{cde}	491±42^{ab}
	0.5	444±35^{bc}	55±5^{bc}	0.39±0.010^{abc}	340±14	871±95^{e}	363±29^{b}
	1	798±140^{a}	95±17^{ab}	0.38±0.007^{abcd}	512±108	1814±401^{bcde}	694±118^{a}
	10	630±53^{abc}	73±6^{abc}	0.37±0.010^{abcd}	455±16	1447±173^{cde}	537±51^{ab}
		588±40^{B}	68±5^{B}	0.368±0.006^{BC}	399±25	1273±113^{B}	506±36^{B}
	0.1	665±45^{ab}	78±6^{a}	0.373±0.007^{b}	421±27	1688±185^{a}	564±38^{ab}
	0.5	559±40^{b}	69±5^{a}	0.392±0.007^{a}	414±22	1451±198^{a}	456±33^{c}
	1	706±37^{a}	81±4^{a}	0.365±0.006^{b}	464±28	1741±116^{a}	609±32^{a}
	10	586±35^{b}	67±4^{a}	0.367±0.005^{b}	450±14	1358±124^{a}	494±33^{bc}

*In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test.

GS and GOGAT activity

Treatment significantly influenced both roots ($P \leq 0.001$) and leaves GS enzyme activity ($P \leq 0.001$) (Table 5). Among treatments, HS_4 and HS_B were the most effective in roots ($P \leq 0.05$), whereas HS_4, HS_A, and HS_USA were the most active in leaves ($P \leq 0.05$). In particular, HS_4 always showed high values in GS enzyme activity, up to 1.63 fold higher than untreated roots (1 mg C L^{-1}) ($P \leq 0.05$), and 1.49 fold higher than untreated leaves (10 mg C L^{-1}) ($P \leq 0.05$). The activity of GOGAT enzyme was also affected by treatment in both roots ($P \leq 0.001$) and leaves ($P \leq 0.001$) (Table 5). In roots, strong effects were induced by HS_USA and HS_4, and in leaves by HS_B. In fact, GOGAT activity was 1.97 and 1.87 fold higher than untreated roots (HS_USA 0.1 C mg L^{-1}) ($P \leq 0.05$) and leaves (HS_B 1 mg C L^{-1}) ($P \leq 0.05$), respectively (treatment × concentration interaction significant at $P \leq 0.05$).

Table 5 Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities of leaves and roots per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, *n*=9

Treatment	Dose (mg C L^{-1})	GS (μmol GHA g^{-1} fw min^{-1})		GOGAT (nmol NADH g^{-1} fw min^{-1})	
		Roots	Leaves	Roots	Leaves
Control	0	$0.101\pm0.008^{cdefCc*}$	0.090 ± 0.004^{cdCc}	0.36 ± 0.05^{bcB}	0.08 ± 0.08^{bcBC}
HS_USA	0.1	0.098 ± 0.009^{cdef}	0.102 ± 0.008^{abcd}	0.71 ± 0.01^{a}	0.09 ± 0.09^{bc}
	0.5	0.084 ± 0.005^{def}	0.110 ± 0.007^{abcd}	0.59 ± 0.02^{ab}	0.05 ± 0.05^{c}
	1	0.091 ± 0.001^{cdef}	0.102 ± 0.006^{abcd}	0.56 ± 0.03^{ab}	0.07 ± 0.07^{bc}
	10	0.135 ± 0.003^{abc}	0.119 ± 0.007^{abc}	0.62 ± 0.05^{ab}	0.07 ± 0.07^{bc}
		0.102 ± 0.004^{C}	0.108 ± 0.003^{B}	0.62 ± 0.02^{A}	0.07 ± 0.005^{C}
HS_4	0.1	0.148 ± 0.008^{ab}	0.126 ± 0.004^{ab}	0.63 ± 0.08^{ab}	0.10 ± 0.10^{b}
	0.5	0.157 ± 0.005^{ab}	0.128 ± 0.010^{ab}	0.61 ± 0.09^{ab}	0.10 ± 0.10^{b}
	1	0.165 ± 0.012^{a}	0.119 ± 0.008^{abc}	0.65 ± 0.06^{a}	0.07 ± 0.07^{bc}
	10	0.155 ± 0.007^{ab}	0.134 ± 0.008^{a}	0.62 ± 0.06^{ab}	0.10 ± 0.10^{b}
		0.156 ± 0.004^{A}	0.127 ± 0.003^{A}	0.63 ± 0.03^{A}	0.09 ± 0.005^{B}
HS_A	0.1	0.085 ± 0.008^{def}	0.097 ± 0.004^{bcd}	0.13 ± 0.00^{c}	0.08 ± 0.08^{bc}
	0.5	0.073 ± 0.001^{ef}	0.108 ± 0.008^{abcd}	0.13 ± 0.03^{c}	0.10 ± 0.10^{b}
	1	0.057 ± 0.008^{f}	0.108 ± 0.003^{abcd}	0.12 ± 0.00^{c}	0.08 ± 0.08^{bc}
	10	0.098 ± 0.018^{cdef}	0.119 ± 0.003^{abc}	0.08 ± 0.03^{c}	0.08 ± 0.08^{bc}
		0.078 ± 0.006^{D}	0.108 ± 0.003^{B}	0.12 ± 0.01^{C}	0.08 ± 0.004^{BC}
HS_B	0.1	0.125 ± 0.001^{abcd}	0.088 ± 0.005^{cd}	0.15 ± 0.01^{c}	0.12 ± 0.12^{ab}
	0.5	0.115 ± 0.002^{bcde}	0.102 ± 0.001^{abcd}	0.17 ± 0.02^{c}	0.14 ± 0.14^{a}
	1	0.119 ± 0.003^{bcde}	0.083 ± 0.007^{d}	0.20 ± 0.01^{c}	0.15 ± 0.15^{a}
	10	$0.131\pm0.011_{abcd}$	0.092 ± 0.002^{cd}	0.18 ± 0.01^{c}	0.14 ± 0.14^{a}
		0.122 ± 0.003^{B}	0.091 ± 0.002^{C}	0.18 ± 0.00^{C}	0.14 ± 0.004^{A}
	0.1	0.114 ± 0.006^{b}	0.103 ± 0.004^{b}	0.40±0.07	0.09±0.006
	0.5	0.107 ± 0.007^{c}	0.112 ± 0.004^{a}	0.38±0.06	0.09±0.009
	1	0.108 ± 0.008^{c}	0.103 ± 0.004^{b}	0.38±0.06	0.09±0.010
	10	0.130 ± 0.006^{a}	0.116 ± 0.004^{a}	0.37±0.06	0.10±0.007

* In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test.

Proteins and soluble sugars content

The treatment with HS affected the proteins content and concentration ($P \leq 0.05$) of maize plants ($P \leq 0.005$) (Table 6). In roots, HS_4 and HS_USA had the highest effects at 1 and 10 mg L^{-1} ($P \leq 0.05$) highlighting values 1.81 and 1.73 fold untreated, respectively. In leaves only HS_A at 0.5 mg C L^{-1} increased the protein content (1.19 fold untreated) ($P \leq 0.05$).

Sucrose content was considerably influenced by treatments ($P \leq 0.005$) (Table 7). This led a general decrease in sugars with respect to the control, however for HS_4 the effect was the opposite at low doses (0.1 and 0.5 mg C L^{-1}), with sucrose content increasing up to 3.39 and 2.07 fold compared to untreated plants, respectively. Fructose content was positively influenced by HS treatment ($P \leq 0.05$). Whereas for glucose content HS treatment had little effect. Although not statistically significant, the trend showed that HS_B induced a widespread increase in the amount of both glucose and fructose.

Table 6 Roots and leaves protein content per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, n=9

Treatment	Dose (mg C L^{-1})	Protein (mg g fw^{-1})	
		Roots	Leaves
Control	0	$0.99\pm0.07^{bCb*}$	8.56 ± 0.34^{bcABbc}
HS_USA	0.1	1.16 ± 0.06^{b}	8.23 ± 0.50^{cd}
	0.5	1.09 ± 0.04^{b}	7.56 ± 0.26^{cd}
	1	1.52 ± 0.36^{ab}	9.37 ± 0.35^{a}
	10	1.72 ± 0.11^{a}	7.16 ± 0.36^{d}
		1.37 ± 0.10^{B}	8.08 ± 0.24^{B}
HS_4	0.1	1.54 ± 0.12^{ab}	8.82 ± 0.27^{b}
	0.5	1.58 ± 0.02^{ab}	9.08 ± 0.34^{ab}
	1	1.79 ± 0.02^{a}	9.06 ± 0.15^{ab}
	10	1.61 ± 0.09^{ab}	8.72 ± 0.63^{bc}
		1.63 ± 0.04^{A}	8.92 ± 0.18^{A}
HS_A	0.1	1.19 ± 0.12^{b}	8.33 ± 0.26^{cd}
	0.5	1.11 ± 0.07^{b}	10.2 ± 0.13^{a}
	1	1.17 ± 0.15^{b}	9.95 ± 0.08^{a}
	10	1.28 ± 0.06^{ab}	7.68 ± 0.12^{cd}
		1.19 ± 0.05^{C}	9.06 ± 0.23^{A}
HS_B	0.1	1.13 ± 0.03^{b}	8.43 ± 0.17^{abcd}
	0.5	1.01 ± 0.05^{b}	8.57 ± 0.25^{bc}
	1	1.02 ± 0.03^{b}	8.72 ± 0.25^{bc}
	10	1.09 ± 0.04^{b}	7.80 ± 0.43^{cd}
		1.06 ± 0.02^{C}	8.38 ± 0.15^{AB}
	0.1	1.25 ± 0.05^{ab}	8.45 ± 0.16^{abc}
	0.5	1.20 ± 0.05^{ab}	8.87 ± 0.23^{ab}
	1	1.37 ± 0.11^{a}	9.28 ± 0.14^{a}
	10	1.42 ± 0.06^{a}	7.84 ± 0.23^{c}

* In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test.

Table 7 Sucrose, glucose, and fructose amount in leaf per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, *n*=9

Treatment	Dose (mg C L^{-1})	Sucrose	Glucose (mg kg^{-1} fw)	Fructose
Control	0	611±288^{AB*}	5820±485	1978±185
HS_USA	0.1	159±14	6273±580	2334±52
	0.5	130±67	6242±637	2403±290
	1	143±4	5999±604	2211±254
	10	143±14	5520±221	2427±124
		144±13^{B}	6009±233	2343±83
HS_4	0.1	2073±472	5833±210	1666±125
	0.5	1266±404	6265±165	2034±47
	1	529±509	4973±803	1609±200
	10	88±40	6297±636	2895±184
		989±323^{A}	5842±284	2051±202
HS_A	0.1	162±16	6099±137	2347±858
	0.5	92±10	5334±434	2325±428
	1	145±13	5287±568	2048±334
	10	174±36	5453±678	2254±320
		143±14^{B}	5543±343	2243±206
HS_B	0.1	160±10	6123±800	2408±257
	0.5	193±26	7792±761	3153±169
	1	219±61	7128±520	2933±354
	10	210±59	5945±452	2192±171
		195±19^{B}	6747±376	2672±174
	0.1	639±325	6082±328	2189±206
	0.5	420±200	6408±392	2479±187
	1	259±114	5847±394	2200±211
	10	154±22	5804±237	2442±131

* In the same column differences among treatment mean (capital letters) were at $P \leq 0.05$ by Student Newman Keuls test.

PAL activity, soluble phenols content, and phenolic acids

PAL activity in maize leaves was significantly influenced by treatment ($P \leq 0.001$) and concentration ($P \leq 0.001$) (Table 8). HS_4, in the range at 0.5 and 1 mg C L^{-1} gave the highest PAL activity. In particular, at 0.5 mg C L^{-1} the PAL activity increased up to 3.70 fold relative to the control (treatment × concentration interaction significant at $P \leq 0.01$). PAL activity was also increased by HS_A at 1 mg C L^{-1} up to 1.54 fold compared to untreated. A general increase of soluble phenolic content was induced by HS treatment ($P \leq 0.001$) (Table 8)with HS_4 and HS_A showing the highest effects. In particular, HS_A at 10 mg C L^{-1} and 0.5 mg C L^{-1} showed a strong effect in the content of *p*-hydroxybenzoic acid (3.25 fold untreated) and *p*-coumaric acid (2.3 fold untreated), respectively. Finally, HS_USA at 1 mg C L^{-1} and HS_B at 10 mg C L^{-1} increased the chlorogenic acid (1.21 fold compared to untreated) (Table 8).

Linear and logarithmic regression curves were tested and they did not explain the data distribution of the enzymes activities, phenols, phenolic acids, proteins, and sugars contents. Parameters (R^2 and *P* value) are reported in Table S1.

Table 8 Phenylalanine ammonia-lyase activity (PAL), soluble phenols content, and phenolic acid profile per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, *n*=9.

Treatment	Dose	PAL	Soluble phenols	Phenolic acid (mg kg^{-1} fw)			
	(mg C L^{-1})	(nmol cinn. acid mg^{-1} prot min^{-1})	(mg gallic acid g^{-1} fw)	Chlorogenic	*p*-Cumaric	Ferulic	*p*-Hydroxybenzoic
Control	0	$2.52\pm0.25^{bC*}$	15.8 ± 0.2^{bC}	80±5	4.3±0.3	5.0±0.9	12 ± 3^{cB}
HS_USA	0.1	1.96 ± 0.53^{b}	18.5 ± 0.3^{a}	88±1	5.0±0.4	6.0±1.0	10±2
	0.5	2.15 ± 0.47^{b}	15.7 ± 0.2^{b}	96±2	5.4±0.1	7.0±1.6	13±0
	1	2.80 ± 0.55^{b}	16.0 ± 0.2^{b}	97±1	4.8±0.1	5.0±1.3	18±0
	10	2.43 ± 0.80^{b}	15.4 ± 0.1^{b}	11±2	5.7±1.2	5.0±0.6	24±6
		2.34 ± 0.28^{C}	16.4 ± 0.3^{C}	98±8	5.2±0.2	6.2±0.5	16 ± 2^{AB}
HS_4	0.1	3.19 ± 0.57^{b}	19.7 ± 0.4^{a}	82±1	4.3±1.0	8.0±2	13±0
	0.5	9.32 ± 0.60^{a}	18.0 ± 0.2^{a}	73±6	4.0±0.8	6.0±2.4	nd
	1	8.17 ± 0.55^{a}	19.6 ± 0.4^{a}	90±7	3.8±1.0	6.0±1.8	nd
	10	7.60 ± 0.39^{a}	19.6 ± 0.3^{a}	70±0	4.2±0.3	5.0±1.6	nd
		7.07 ± 0.73^{A}	19.2 ± 0.23^{A}	79±5	4.1±0.3	6.6±0.8	13 ± 0^{B}
HS_A	0.1	3.60 ± 1.19^{b}	19.1 ± 0.5^{a}	79±2	3.6±0.1	2.0±0.2	25±2
	0.5	3.70 ± 0.52^{b}	18.8 ± 0.6^{a}	47±1	10±6.7	6.0±4.1	9±3
	1	3.88 ± 0.82^{b}	18.5 ± 0.2^{a}	84±1	4.2±0.0	2.0±0.2	29±1
	10	2.80 ± 0.69^{b}	19.9 ± 0.4^{a}	83±1	4.0±0.0	2.0±0.4	39±6
		3.50 ± 0.40^{B}	19.1 ± 0.24^{A}	73±7	5.5±1.6	3.4±0.9	26 ± 4^{A}
HS_B	0.1	2.18 ± 0.18^{b}	16.0 ± 0.3^{b}	71±9	4.3±0.4	4.0±0.6	16±3
	0.5	1.46 ± 0.48^{b}	18.7 ± 0.3^{a}	93±2	5.2±0.8	6.0±1.9	23±0
	1	2.57 ± 0.61^{b}	18.6 ± 0.8^{a}	94±5	4.6±0.0	5.0±0.1	29±2
	10	1.69 ± 0.38^{b}	16.7 ± 0.4^{b}	97±0	4.9±0.0	6.0±0.5	28±5
		1.97 ± 0.22^{C}	17.5 ± 0.34^{B}	89±6	4.8±0.2	5.5±0.5	24 ± 2^{A}
	0.1	2.67 ± 0.39^{b}	18.3±0.3	80±6	4.3±0.2	5.3±0.9	$14.\pm3.0^{ab}$
	0.5	3.42 ± 0.62^{a}	17.8±0.3	77±1	6.2±1.5	6.8±1.0	$11.\pm3.2^{b}$
	1	3.81 ± 0.52^{a}	18.2±0.3	91±3	4.4±0.2	4.7±0.6	$19.\pm5.0^{ab}$
	10	3.06 ± 0.52^{ab}	17.9±0.4	90±7	4.7±0.3	5.0±0.5	$23.\pm5.8^{a}$

nd, not detected

* In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test

Discussion

Several studies have reported possible relationships between the effects of leonardite on plant growth and their capacity to improve nutrient uptake and assimilation (Aguirre et al. 2009; Tahiri et al. 2015). Ertani et al. (2011) demonstrated that, in maize, HS from leonardite enhanced the production of N assimilates and promoted photosynthesis through the increase in chlorophyll content and stimulation of RuBisCo enzyme activity. These effects were mainly attributed to a complex macromolecular system mainly composed of polyaromatic rings, and may depend on the origin and characteristics of HS (Nardi et al. 2009).

This study showed that four different HS from leonardites had the same main functional components, but deconvolution fitting analysis gave more information on semi-quantitative differences between leonardites. In terms of functional groups distribution, HS_USA had the highest content in carboxyl groups bound to aromatic rings with few polar substituents. In contrast, HS_4 and in particular in HS_A and HS_B, the aromatic component was dominant and was composed by polar substituents and aliphatic structures. This feature has been related to low rank coals. Overall, HS differently but significantly affected the growth of watercress root and lettuce seedlings in a logarithmic curve model. These effects are reported to reflect strong biostimulant properties (Scaglia et al. 2016). However, the magnitude and nature of these effects were different depending on the type of leonardite. IAA-like activity decreased in the order HS_A > HS_USA > HS_4, whereas HS_USA and HS_A had the highest and lowest GA-like activity, respectively. These results partially agree with those of Ertani et al. (2011) who found GA-like activity in a humic acid from leonardite, while other authors reported auxin-like properties (O'Donnell 1973). However, the intensities of IAA and GA-like activities were comparable with those found from humic and fulvic acids extracted from soil of natural ecosystems (Pizzeghello et al. 2015) and HS extracted from vermicompost (Scaglia et al. 2016).

The relationship between the biological activity and the structure of HS is very important to understand their effects in treated plants (Muscolo et al. 2007). However, the molecular structure is still debated. Recently, HS are described as supramolecular structures formed by relatively small molecules held together by non-covalent intermolecular interactions (e.g., hydrogen-bonding, charge-transfer, van der Waals and π-π) (Piccolo 2001). The exterior domain consists of polar groups (e.g., carboxylic acids) where their distribution is particularly

relevant to determine HS solubility and biological reactivity (Muscolo et al. 2007). High content of carboxylic acids, proteins, and amino acids has been related to IAA-like activity. The carboxyl groups presence is a proxy for the bioavailability of auxin entrapped into the HS molecular structure (Napier 2004). Whereas the hydrophobic domain, composed by aromatics and amides functional groups, is related to GA-like activity (Pizzeghello et al. 2015). Such distinction might not always be applicable and it is possible that leonardite properties might be modified according to the functional groups exposed to the surrounding aqueous environment (Carletti et al. 2010). Nevertheless, our FT-IR spectra of HS confirmed a different content in carboxyl and aromatics rings that may justify their biological activity.

The most general trait in plant responses to HS pertains to growth and architecture of the rooting system, mainly affecting lateral root formation (Canellas and Olivares 2014; Nardi et al. 2009). Rooting is vital for plant survival in relation to nutrition and growth requirements, synthesis and accumulation of secondary metabolites and interaction with nitrogen-fixing organisms (Saini et al. 2013). Root is also the first plant organ targeted by HS in soil. In this study, among the leonardites, HS_USA had the strongest effect in maize root architecture, leading to an overall stimulation of elongation and proliferation of secondary roots as well increasing root diameter. HS_A and HS_USA, which showed high auxin-like activity in the bioassay, showed the highest morphological changes on maize root apparatus, resulting in a higher root surface area, increase of total radicular length and root diameter. This confirms the effect of the carboxylic components in HS. Primary root elongation, and increasing lateral roots are known to be an auxin-triggered mechanism (De Smet et al. 2006), which has been recently proven to be driven by the auxin entrapped in the HS themselves (Trevisan et al. 2010a). Effects on root architecture are indeed accompanied by changes in the biochemistry of energy generation and transport system across plasma membranes (Canellas et al. 2002; Zandonadi et al. 2007). Regarding root diameter, our results agree with previous findings which demonstrated that HS induced a higher rate of differentiation of cells of the root central cylinder relative to untreated plants. The augmented thickness of cells wall has been shown to be due to a higher production of lignin in HS-treated plants (Concheri et al. 1996; Nardi et al. 2000).

HS increased the enzymes involved in N assimilation (Baglieri et al. 2014). In particular, GS and GOGAT enzyme activities were widely affected by the presence of HS. These enzymes work in close association as the incorporation of ammonium (NH_4^+) into organic compounds

by GS leads to the production of glutamate from glutamine and α-ketoglutarate by GOGAT. The GS/GOGAT system is the main metabolic route for N assimilation in higher plants (Mokhele et al. 2012), and its stimulation confirms the capability of HS from leonardites, to interact with the plant nitrate metabolism inducing an increase in N organic compounds, as supported by the augmented protein content recorded. Such results are consistent with previous ones, obtained with HS from other sources such as earthworms coprolites and lignosulfonate-humates (Carletti et al., 2008; Ertani et al. 2011).

In addition to nitrogen metabolism, HS may modulate C metabolism by increasing the activity of enzymes involved in glycolysis and the Krebs cycle (Nardi et al. 2007). In our study, after the application of HS from leonardites, the content of carbohydrates, such as glucose and fructose, sharply increased in the leaves. Carbohydrates, which represent the basis of plant metabolism (Winter and Huber 2000), not only provide the energy required for various metabolic pathways, but also provide carbon skeletons for nitrogen metabolism, thus their increase may justify the improved activity of nitrogen assimilation.

The activity of PAL, synthesized in response to HS treatment, results in the accumulation of phenolic compounds. PAL is an enzyme which, catalyzing the first metabolic step from primary to secondary metabolism (Douglas 1996), deaminates phenylalanine to produce cinnamic acid. As a consequence, HS_4 and HS_A enhanced the soluble phenols and strongly increased phenolics such *p*-hydroxybenzoic acid, *p*-coumaric acid and chlorogenic acid. The stimulation of secondary metabolism is also justified by the enhanced activity of primary metabolism. In addition, a greater concentration of phenols recorded in plants after treatment with leonardites is likely to be the result of a weak uncoupling of oxidative phosphorylation, which in turn increases the metabolic processes requiring glucose (Muscolo and Sidari 2006).

In conclusion, with this work, we aimed to test and compare the biostimulant activity of HS extracted from different leonardites. Overall, HS from leonardites positively affected root architecture, with a stimulation of the elongation and proliferation of secondary roots. They enhanced plant nutrient uptake and nutrient use efficiency, and influenced N metabolism, increasing GOGAT and GS enzymes activity, and hence protein production. The carboxyl groups resulted as proxy for the bioavailability of the auxin entrapped into the HS molecular structure, whereas the hydrophobic domain is related to GA-like activity. From this study it was found that: (1) HS from leonardites have strong biostimulant properties, (2) the leonardite

origin has an influence on composition of the main functional groups, and, as a consequence, on their biological activity.

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Supplementary material

Table S1 Parameters (R^2 and *P* value) of linear and logarithmic regression analyses between concentrations and studied parameters of maize seedlings treated with humic substances (HS) from leonardites. Data are subdivided for HS.

		Linear		Logarithmic	
Treatment	Parameter	R^2	*P* value*	R^2	*P* value
HS_USA	TRL	0.063	0.103	0.400	0.001
	Area	0.039	0.203	0.430	0.001
	Diameter	0.045	0.170	0.012	0.486
	Tips	0.001	0.861	0.014	0.449
	Forks	0.015	0.496	0.400	0.001
	Thin Roots	0.072	0.083	0.450	0.000
	GS roots	0.142	0.008	0.005	0.626
	GS leaves	0.131	0.011	0.194	0.002
	GOGAT roots	0.017	0.383	0.082	0.048
	GOGAT leaves	0.001	0.867	0.012	0.453
	Proteins roots	0.210	0.001	0.203	0.001
	Proteins leaves	0.084	0.046	0.035	0.201
	Sucrose	0.032	0.511	0.153	0.134
	Glucose	0.019	0.607	0.003	0.831
	Fructose	0.067	0.332	0.188	0.093
	PAL	0.142	0.008	0.003	0.728
	Soluble phenols	0.131	0.011	0.016	0.390
	Chlorogenic acid	0.017	0.383	0.211	0.074
	p-Cumaric acid	0.001	0.867	0.251	0.048
	Ferulic acid	0.210	0.001	0.043	0.440
	p-Hydroxybenzoic acid	0.084	0.046	0.193	0.116

HS_4	TRL	0.033	0.234	0.208	0.002
	Area	0.019	0.366	0.139	0.012
	Diameter	0.054	0.126	0.184	0.003
	Tips	0.066	0.089	0.078	0.063
	Forks	0.020	0.360	0.116	0.022
	Thin Roots	0.034	0.225	0.226	0.001
	GS roots	0.086	0.043	0.438	0.001
	GS leaves	0.156	0.005	0.453	0.000
	GOGAT roots	0.016	0.388	0.085	0.044
	GOGAT leaves	0.004	0.680	0.000	0.903
	Proteins roots	0.104	0.026	0.536	0.000
	Proteins leaves	0.000	0.951	0.016	0.390
	Sucrose	0.106	0.220	0.048	0.174
	Glucose	0.022	0.587	0.002	0.760
	Fructose	0.391	0.010	0.083	0.071
	PAL	0.086	0.043	0.443	0.000
	Soluble phenols	0.156	0.005	0.683	0.000
	Chlorogenic acid	0.016	0.388	0.018	0.405
	p-Cumaric acid	0.004	0.68	0.013	0.489
	Ferulic acid	0.104	0.026	0.000	0.985
	p-Hydroxybenzoic acid	0.000	0.951	0.266	0.003

HS_A	TRL	0.010	0.527	0.161	0.007
	Area	0.014	0.443	0.174	0.005
	Diameter	0.020	0.363	0.019	0.368
	Tips	0.011	0.507	0.131	0.016
	Forks	0.393	0.017	0.110	0.028
	Thin Roots	0.010	0.514	0.131	0.016
	GS roots	0.003	0.735	0.084	0.045
	GS leaves	0.158	0.005	0.231	0.001
	GOGAT roots	0.051	0.121	0.170	0.004
	GOGAT leaves	0.001	0.845	0.000	0.982
	Proteins roots	0.057	0.103	0.096	0.032
	Proteins leaves	0.063	0.085	0.021	0.331
	Sucrose	0.025	0.558	0.151	0.0136
	Glucose	0.009	0.722	0.021	0.596
	Fructose	0.012	0.691	0.055	0.380
	PAL	0.003	0.735	0.070	0.069
	Soluble phenols	0.158	0.005	0.681	0.000
	Chlorogenic acid	0.051	0.121	0.026	0.550
	p-Cumaric acid	0.001	0.845	0.031	0.517
	Ferulic acid	0.057	0.103	0.112	0.205
	p-Hydroxybenzoic acid	0.063	0.085	0.377	0.019

HS_B	TRL	0.063	0.085	0.229	0.001
	Area	0.091	0.044	0.193	0.003
	Diameter	0.006	0.625	0.042	0.178
	Tips	0.050	0.140	0.005	0.636
	Forks	0.092	0.043	0.123	0.018
	Thin Roots	0.095	0.040	0.243	0.001
	GS roots	0.100	0.034	0.125	0.014
	GS leaves	0.091	0.044	0.002	0.762
	GOGAT roots	0.006	0.625	0.094	0.034
	GOGAT leaves	0.050	0.140	0.088	0.040
	Proteins roots	0.092	0.043	0.015	0.409
	Proteins leaves	0.095	0.040	0.008	0.542
	Sucrose	0.022	0.582	0.112	0.185
	Glucose	0.003	0.836	0.124	0.181
	Fructose	0.000	0.944	0.308	0.026
	PAL	0.100	0.034	0.057	0.103
	Soluble phenols	0.091	0.044	0.269	0.000
	Chlorogenic acid	0.006	0.625	0.102	0.229
	p-Cumaric acid	0.050	0.140	0.100	0.233
	Ferulic acid	0.092	0.043	0.007	0.756
	p-Hydroxybenzoic acid	0.095	0.040	0.533	0.003

* significant at $P < 0.05$

TRL = total root length; GS = glutamine synthetase; GOGAT = glutamate synthase; PAL = phenylalanine ammonia-lyase

6 Manuscript IV

Biostimulant effects of seed-applied sedaxane fungicide: morphological and physiological changes in maize seedlings

Dal Cortivo C. [1#], Conselvan G.B. [1#], Carletti P. [1*], Barion G. [1], Sella L.[2], Vamerali T. [1]

[1] Department of Agronomy, Food, Natural Resources, Animals and the Environment, University of Padua, Padua, Italy

[2] Department of Land, Environment, Agriculture and Forestry, University of Padua, Padua, Italy.

* Corresponding author. E-mail address: paolo.carletti@unipd.it

[#] These authors contributed equally to this work.

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Abstract

Most crops are routinely protected against seed-born and soil-borne fungal pathogens through seed-applied fungicides. The recently-released succinate dehydrogenase inhibitor (SDHI), sedaxane®, is a broad-spectrum fungicide, used particularly to control *Rhizoctonia* spp., but also has documented growth-enhancement effects on wheat. This study investigates the potential biostimulant effects of sedaxane and related physiological changes in disease-free maize seedlings (3-leaf stage) at increasing application doses (25, 75 and 150 μg a.i. seed^{-1}) under controlled sterilized conditions.

We show sedaxane to have significant auxin-like and gibberellin-like effects, which effect marked morphological and physiological changes according to an approximate saturation dose-response model. Maximum benefits were attained at the intermediate dose, which significantly increased root length (+60%), area (+45%) and forks (+51%), and reduced root diameter as compared to untreated controls. Sedaxane enhanced leaf and root glutamine synthetase (GS) activity resulting in greater protein accumulation, particularly in the above-ground compartment, while glutamate synthase (GOGAT) activity remained almost unchanged. Sedaxane also improved leaf phenylalanine ammonia-lyase (PAL) activity, which may be responsible for the increase in shoot antioxidant activity (phenolic acids), mainly represented by *p*-coumaric and caffeic acids.

We conclude that, in addition to its protective effect, sedaxane can facilitate root establishment and intensify nitrogen and phenylpropanoid metabolism in young maize plants, and may be beneficial in overcoming biotic and abiotic stresses in early growth stages.

Keywords

Biostimulant; hormone-like activity; nitrogen metabolism; phenolic acids; root branching; Succinate Dehydrogenise Inhibitor (SDHI)

Introduction

In intensive agriculture, seed coating is a technique of applying several compounds, such as pesticides, fertilizers and biostimulant substances, to the seed surface so they can start to act on the seedlings during germination and/or at the seed-soil interface immediately after sowing (Ehsanfat and Modarres-Sanavy 2005).

Protecting field crop plants from soil- and seed-borne pathogens during germination and in early growth stages is crucial to ensure safe and fast establishment (Mathre et al. 2001). Fungicides are chemical and biological compounds that kill pathogenic fungi or inhibit fungal spore germination (McGrath 2004), and, together with insecticides, are the molecules most frequently used in the seed coatings of many crops.

A fungicidal seed treatment is commonly composed of a trace quantity of fungicide evenly distributed among the seeds along with the adhesive substances needed to bind them to the seed surface (Sharma et al. 2015). Modern seed dressing fungicide formulations are often a mixture of several active ingredients with different modes of action (systemic and contact), which broadens the spectrum of control to include a wide range of pathogens and reduces the likelihood of resistance onset (Kitchen et al. 2016). Common fungicide combinations for cereals are triticonazole + prochloraz (Krzyzinska et al. 2005; Vermeulen et al. 2017), both sterol-inhibiting fungicides, and fludioxonil + metalaxyl-M (Mondal 2004), the former a non-systemic phenylpyrrole, which inhibits transport-associated phosphorylation of glucose, the latter an acilalanine RNA synthesis inhibitor.

Substances on the seed surface can affect germination, as they may vary considerably in the degree to which they attract or repel moisture (Scott 1989). When applied in high concentrations, fungicides have been reported to have potential direct negative effects on seed germination, rootlet growth, and emergence (Minamor 2013). In many cases, the effects of seed-applied fungicides on plants vary according to growing conditions: under low pathogen pressure, they do not improve crop emergence and grain yield of wheat, but under high pressure from *Fusarium graminearum* they do (May et al. 2010). Environmental factors may also play a role (Cox and Cherney 2014). Seed coating is expected to suppress arbuscular mycorrhizal fungi, hindering their colonization of roots and consequently reducing their beneficial effects on plant growth (Channabasava et al. 2015; Chiocchio et al. 2000).

In the search for highly effective active ingredients, attention is currently focused on useful secondary effects of fungicides on seedling development, regardless of genotype and growing conditions. Several fungicides have been found to have positive side-effects on plant

physiology (Berdugo et al. 2012): The ubiquinol oxidase inhibitor (Qol) Strobilurin family is known to increase several morphological traits of maize, such as leaf number and area, and shoot and root biomasses (Lazo and Ascencio 2014). Strobilurins have also been found to increase tolerance to abiotic stresses, as they can delay senescence of the photosynthetic leaf area, change the balance of the phytohormones, and increase CO_2 assimilation in wheat (Köhle et al. 2002; Wu and von Tiedemann 2001). The azole fungicide class also influences the physiology of treated plants by increasing the chlorophyll content in winter wheat plants, delaying leaf senescence, and protecting plants from several abiotic stresses (Fletcher et al. 2010).

Recent studies have demonstrated the influence of pyrazole-carboxamide succinate dehydrogenase inhibitors (SDHIs) on plant physiology (Ajigboye et al. 2016; Ajigboye et al. 2014). These comprise a relatively new class of fungicide (since 2000), and now include various active ingredients, such as boscalid, bixafen, isopyrazam and sedaxane, which can disrupt fungal respiration causing a breakdown in energy/ATP production (Avenot and Michailides 2010). The SDHI sedaxane (Syngenta Crop Protection, Basel, Switzerland) has recently been released for use as a treatment for local and systemic protection of cereal seeds, seedlings and roots against pathogenic fungi, both seed-borne (*Ustilago nuda, Tilletia caries, Monographella nivalis, Pyrenophora graminea*) and soil-born (*Rhizoctonia solani, R. cerealis, Gaeumannomyces graminis*, *Typhula incarnata*) (Ajigboye et al. 2016; Zeun et al. 2013). When sedaxane moves from the seed to the soil and into the plant tissues, it has been found to improve the development of the roots and lower stems of cereals (Swart 2011). Previous research has described wheat responding positively to sedaxane in terms of greater biomass, better growth and drought resistance (Ajigboye et al. 2016). These morpho-physiological reactions are also known to be induced by biostimulants (Calvo et al. 2014), defined as substances that at low doses are able to enhance hormone biosynthesis, nutrient uptake from the soil, resistance to biotic/abiotic stresses, crop quality, and root growth (Kauffman et al. 2007).

Given all this, the present study aimed to investigate the potential biostimulant activity of seed-applied sedaxane on maize plants, and the possible physiological mechanisms underlying the morphological changes. To this end, we: i) carried out a bioassay (Audus test) to determine the biostimulant activity of sedaxane, ii) measured the morphological variations in pot-cultivated, disease-free maize plants at increasing fungicide doses, and iii) studied the response of the enzymes involved in nitrogen and phenylpropanoid metabolism, and the protein, sugar and total phenol contents in the leaves and roots of the same plants.

Materials and Methods

Characteristics of sedaxane

In this study, we used the fungicide formulation Vibrance® 500 FS, a commercial flowable concentrate for seed treatment containing 500 g sedaxane® L^{-1}, i.e., 43.7% w/w of AI (density 1.17 g mL^{-1}; pH 6.39). Sedaxane is the ISO common name for a mixture of two *cis*-isomers, 2'-[(1RS,2RS)-1,1'-bicycloprop-2- yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide and two *trans*-isomers 2'-[(1RS,2SR)-1,1'- bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide (IUPAC). Its minimum purity is 960 g kg^{-1}, with ranges of 820-890 g kg^{-1} for the 2 *trans*-isomers (SYN508210 - 50:50 mixture of enantiomers), and 100-150 g kg^{-1} for the 2 *cis*-isomers (SYN508211 - 50:50 mixture of enantiomers) (EFSA 2012).

Pot trial set-up and plant analysis

Plants of the maize hybrid Hydro (Syngenta, Basel, Switzerland) were grown in cylindrical PVC pots (50 cm high, 9 cm diameter, 3.1 L volume) in a greenhouse in the L. Toniolo experimental farm of the University of Padua (Legnaro, NE Italy). The pots were filled with a sterilized mixture (36 h in an oven at 120 °C) of silty-loam soil collected from a field on the experimental farm (pH 8.4), and fine sand (1:1 w/w) to facilitate water drainage and root collection, to which was added a standard dose of pre-sowing fertilizer (about 100 kg N ha^{-1}, 150 kg P_2O_5 ha^{-1} and 300 kg K_2O ha^{-1}). Maize seeds were treated with three increasing doses of sedaxane: 25, 75 and 150 μg AI $seed^{-1}$, corresponding to label doses of 2.5, 7.5 and 15 mL of the commercial product Vibrance® 500 FS (500 g AI L^{-1}) in 50,000 seeds. Plants grown from treated seeds were compared with untreated controls. The experimental design was completely randomized with 6 replicates.

Three seeds per pot were sown at the end of June, and immediately after emergence plants were thinned to one per pot. At harvest, growth measurements were taken from three pots/plants, and enzymatic activity assays were carried out with a further three.

Water stress was avoided throughout the experiment by regularly watering the plants. Before plant harvest, which took place 20 days after sowing (DAS) at the 3-leaf stage, SPAD (Soil Plant Analysis Development) was measured in the last fully-developed leaf with a 502 chlorophyll meter (Konica-Minolta, Hong Kong). Fresh and dry (oven-dried for 24 h at 105 °C) weights were measured on three replicate samples of shoots, and roots were collected, gently washed of soil, and stored in a 15% v/v ethanol solution until morphological

characterization. Root length, surface area, diameter, and number of tips and forks were measured by analysis of 1-bit 400-DPI images of the roots acquired with a flatbed scanner (Epson Expression 11000XL, Epson, Suwa, Japan) using the WinRhizo software (Regent Instruments, Ville de Québec, Canada).

Three replicates were stored at -80 °C until analysis, then shoot and root tissue samples were taken from them for enzymatic activity assays. Each enzymatic assay (n = 9) was carried out in triplicate on each plant.

A further trial was performed following the same procedure and timing of the main experiment, and using the same sand-soil mixture (1:1 w/w), but this time it was not sterilized. We took SPAD readings, and measured fresh and dry weights, and root morphological parameters of plants grown in unsterilized soil, as reported above (Supplementary Material, Table SM1).

Bioassay to test the biological activity of sedaxane

In order to investigate the biological activity of sedaxane, we measured the reduction in root growth in the model plant watercress (*Lepidium sativum* L.) to assess auxin-like activity, and the increase in shoot length in lettuce (*Lactuca sativa L.*) to assess gibberellin-like activity (Audus 1972).

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 minutes. After rinsing 5 times with sterile distilled water, 20 seeds were aseptically placed on filter paper in a Petri dish. In the case of watercress, the filter paper was moistened with 1.2 mL of H_2O (controls), or with 1.2 mL of 0.1, 1, 10 and 20 mg L^{-1} indoleacetic acid (IAA, natural auxin) (Sigma-Aldrich, St. Louis, MO, USA) to obtain the calibration curve, or with 1.2 mL of a serial dilution of the tested product Vibrance containing 500 g L^{-1} of AI sedaxane. The experimental design for lettuce was the same as for watercress, except that the sterile filter paper was moistened with 1.4 mL of the above solutions, while the calibration curve was a progression of 0.0001, 0.001, 0.01 and 0.1 mg L^{-1} gibberellic acid (GA) (Sigma-Aldrich, St. Louis, MO, USA).

Seeds were germinated in the dark at 25 °C. After 48 h, the watercress seedlings were removed from the dishes and the roots measured with a digital gauge; after 72 h, the lettuce seedlings were removed and the shoots measured with a digital gauge.

A linear regression model ($y = a + bx$) was used to describe the dose-response relationship after logarithmic transformation of IAA, GA and the sedaxane doses, where x was the sedaxane concentration (g L^{-1}) and y the root or shoot length (mm) (Conselvan et al. 2017).

Protein extraction and determination

Fresh leaf and root samples, previously stored at -80 °C, were ground to a homogenous powder with liquid N_2. Proteins were extracted by homogenizing 0.5 g of root or shoot materials with 5 mL of 38 mM KH_2PO_4 and 62 mM K_2HPO_4, pH 7, at 4 °C. After 2 minutes, the extract was filtered through three layers of muslin and centrifuged at 15,000 g for 20 min at 4 °C. A 50 µL supernatant sample was incubated with 50 µL of Milli-Q water and 2.5 mL of 0.00117 M Bradford reagent. After 15 min, the protein concentration in the extract was determined according to Bradford (1976), using a Jasco V-530 UV/Vis spectrophotometer (Jasco Corporation, Tokyo, Japan) at 595 nm wavelength. The protein concentration was expressed as mg of protein per g of fresh root or shoot.

Enzyme extraction and assay conditions

To extract the enzymes involved in N reduction and assimilation pathways, fresh shoot and root samples were ground to a homogeneous powder with liquid N_2. Each activity assay was carried out in triplicate and with 3 biological repetitions using specific buffers for enzyme extraction.

Glutamine synthetase (GS; EC 6.3.1.2) was extracted by homogenizing 0.6 g of root or shoot material at 4 °C with 2.4 mL of a solution of 1 mM Tris(hydroxymethyl)aminomethane HCl (Tris-HCl), 25 mM KH_2PO_4, 10 mM L-cysteine hydrochloride monohydrate and 3% (w/v) bovine serum albumin at pH 7.8 (Baglieri et al. 2014). After 10 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 g for 25 min at 4 °C. A 200 µL sample of supernatant was incubated with 200 µL of reaction buffer (50 mM Tris-HCl, 20 mM $MgSO_4$, 80 mM L-glutamate, 30 mM NH_2OH, 24 mM ATP, pH 7.8) at 37 °C for 25 min. Reaction was blocked with a stopping solution (0.5 mL of 370 mM $FeCl_2 \cdot 6H_2O$ and 670 mM HCl). Samples were centrifuged at 15,000 g for 15 min. The amount of γ-glutamyl hydroxamate in the supernatant was determined photometrically (wavelength 540 nm) against an immediately-stopped parallel sample (Jezek et al. 2015). A standard curve was made using authentic γ-glutamyl hydroxamate (GHA) proportional to absorbance intensity. Enzyme activity was expressed as µmol of GHA produced per g of fresh root or leaf tissue per minute (Conselvan et al. 2017).

Glutamate synthase (GOGAT; EC 1.4.7.1) was extracted by homogenizing 0.5 g of root or shoot material with 2 mL of a solution of 100 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2 \cdot 6H_2O$, 2

mM β-mercaptoethanol, 10% (v/v) glycerol and 1 mM Na_2EDTA. After 15 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was centrifuged a second time at 15,000 g for 15 min at 4 °C. For the enzyme assay, 100 µL of extract was added to 900 µL of reaction buffer (41.6 mM HEPES, pH 7.5, 1 mM NADH, 10 mM EDTA, 20 mM glutamine) and 300 µL (for leaf extract) or 900 µL (for root extract) of 10 mM α-ketoglutaric acid. The reaction time was 2 min for the shoot extract, and 1.5 min for the root extract at 30 °C. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at wavelength 340 nm according to Avila et al. (1987). GOGAT activity was expressed as nmol NADH reduced per g of fresh root or shoot per minute.

For the phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) assay, 1 g of shoot material was homogenized with 0.1 g of poly(vinylpolypyrrolidone) (PVPP) and 5 mL of 100 mM potassium phosphate buffer (pH 8.0) containing 1.4 mM β-mercaptoethanol. After 10 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 g for 20 min at 4 °C. A 60 µL sample of supernatant was incubated with 400 µL of 100 mM Tris-HCl buffer (pH 8.8), 140 µL of 100 mM phosphate buffer and 200 µL of 40 mM phenylalanine, at 37 °C for 30 min. Reaction was stopped with 200 µL 6 M HCl (El-Shora 2002). After centrifuging at 10,000 g for 15 min, the absorbance of the supernatant was measured at 280 nm against an immediately-stopped parallel sample. A standard curve was made using authentic cinnamic acid at increasing dilutions. PAL activity was expressed as nmol cinnamic acid produced per mg of protein in the sample per minute.

Soluble phenols extraction and determination

Soluble phenolic acids were extracted by homogenizing 200 mg of leaf material with 600 mL of pure methanol. The extract was kept on ice for 30 min then centrifuged at 15,000 g for 30 min at 4 °C. Total phenols were measured according to the procedure described by Arnaldos et al. (2001). In brief, 1 mL of 2% Na_2CO_3 and 75 µL of Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) were added to 50 µL of the phenolic extract. After incubation in the dark for 15 min at 25 °C, absorbance was measured at 725 nm. A standard curve was made using authentic gallic acid. The soluble phenol content was expressed as mg of gallic acid equivalent (GAE) per g of fresh shoot material.

Free phenolic acid concentrations were revealed on 0.1 g shoot samples treated with 5 mL 80% (v/v) acetonitrile (ACN) in 10 mL tubes for 5 min at room temperature with agitation (70 rpm). After centrifugation (5 min, 10,000 RCF), clear supernatant was filtered at 0.2 µm

(Acrodisc syringe filters with GHP membranes) and kept in clean tubes at -20 °C until processing. HPLC analysis was carried out according to the method described by Adom et al. (2003) with modifications. Samples were manually shaken, then 200 µL was extracted and placed in vials for HPLC autosampling. The mobile phase was 0.25% (v/v) trifluoroacetic acid (TFA, solvent A) and pure ACN (solvent B). The HPLC gradient was linear: after 2 µL sample injection, solvent B was kept at 4% for 1.16 min, then increased gradually to 12% over 1.16 min, to 23% over 4.63 min, to 95% over 1.85 min, and to 100% over 1.16 min, with final rate maintained for a further 2.78 min. The duration of the analysis was 11.58 min at a solvent flow rate of 1.1 mL min^{-1}. The HPLC equipment (Shimadzu, Kyoto, Japan) had a UV diode array detector (SPD-M20A) at wavelength 282 nm, and an Ultra Tech sphere C18 analytical column (33 mm × 4.6 mm i.d., 1.5 µm particle size; Cil Cluzeau, Sainte-Foy-La-Grande, France) kept at 36 °C. Control sample solutions of shoots containing known phenolic acid concentrations were analyzed at the beginning of each new batch analysis, and measurement accuracy was verified by checking expected concentrations.

Each peak was identified by analyzing the retention time and absorbance spectrum of each pure compound (i.e., *p*-Coumaric, caffeic, syringic, vanillic and *t*-ferulic acids; supplementary Fig. 1). The coefficients of determination of all calibration curves were > 99%.

Quantitative determination of sugars

Shoots (5 g) were homogenized in methanol (20 mL) with an Ultra Turrax T25 at 13,500 rpm for 30 s until they attained uniform consistency. Samples were filtered once through filter paper (589 Schleicher), and a second time through cellulose acetate syringe filters (0.45 µm). The extract was then ready for HPLC analysis, for which we used a Jasco X-LC liquid chromatography system (Jasco Inc., Easton, MD, USA) consisting of a PU-2080 pump, an MD-2015 multiwavelength detector, an AS-2055 autosampler, and a CO-2060 column oven interfaced to a PC using the ChromNAV chromatography data system software (Jasco Inc., Easton, MD, USA).

Sugars were separated in a HyperRez XP Carbohydrate Pbþþ analytical column (7.7 mm × 300 mm; ThermoFisher Scientific, Waltham, MA, USA), operating at 80 °C. Isocratic elution was carried out with water at a flow rate of 0.6 mL min^{-1}. D-(β)-glucose and D-(β)- fructose were quantified by a calibration method. Standards were dissolved in water and the calibration curves were generated with concentrations ranging from 100 mg L^{-1} to 1,000 mg

L^{-1} (Nicoletto et al. 2013).

Statistical analysis

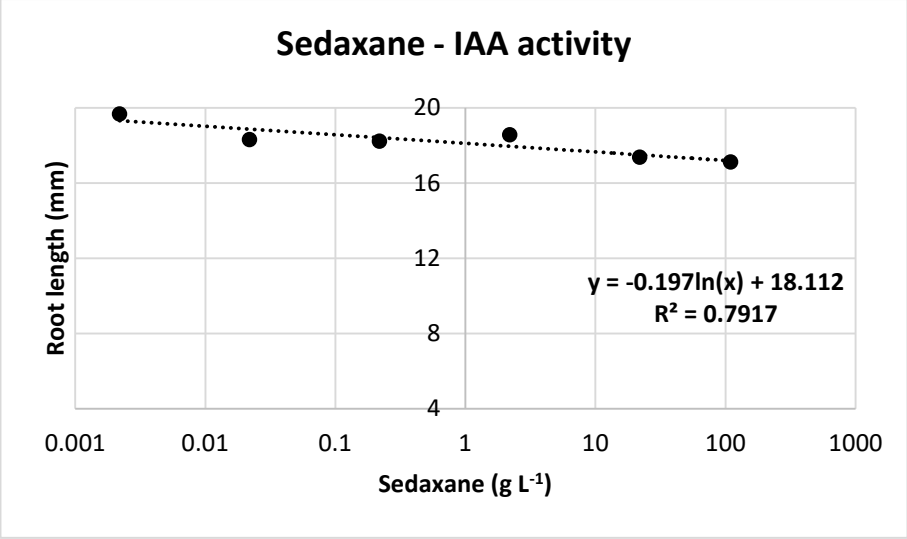
The data are the means of measurements from three different pots per treatment. The analysis of variance (ANOVA) was performed in the SPSS 23 (IBM Corp) software, and was followed by pairwise post-hoc analyses (Student-Newman-Keuls test) to determine significant differences among means at $P \leq 0.05$.

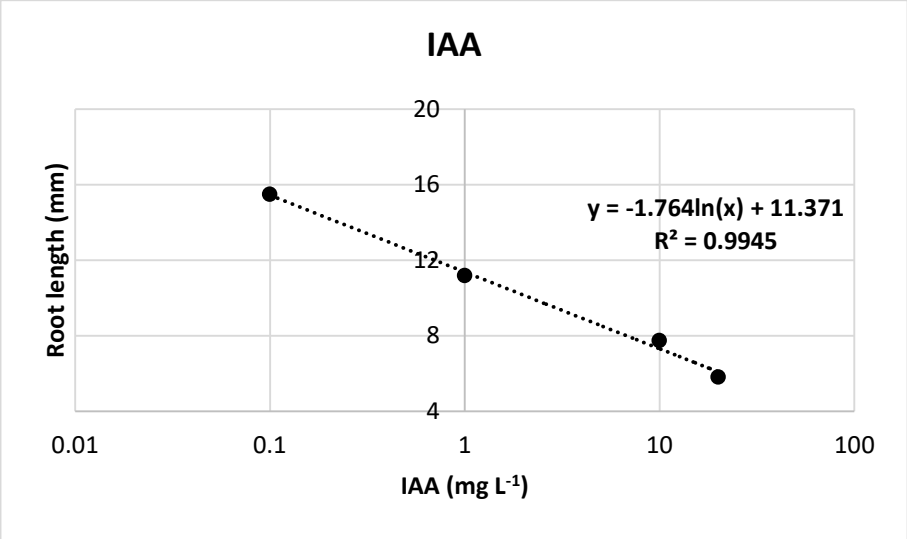
Results

Audus test and effects of sedaxane on shoot and root growth

Ahead of the analysis, the Audus test was used to determine the biostimulant properties of the active ingredient sedaxane. As with the natural auxin IAA, which reduces root elongation in the model plant watercress and is dose-proportional, increasing concentrations of sedaxane led to a progressive reduction in root length, suggesting an auxin-like effect (Fig. 1). We also found sedaxane to exhibit gibberellin-like activity, as it enhanced the shoot growth of lettuce and had a similar dose-proportionality to exogenous gibberellic acid (Fig. 2). Both regression curves were significant ($P < 0.02$ for root responses, $P \leq 0.05$ for shoot responses), revealing the hormone-like activity of this fungicide.

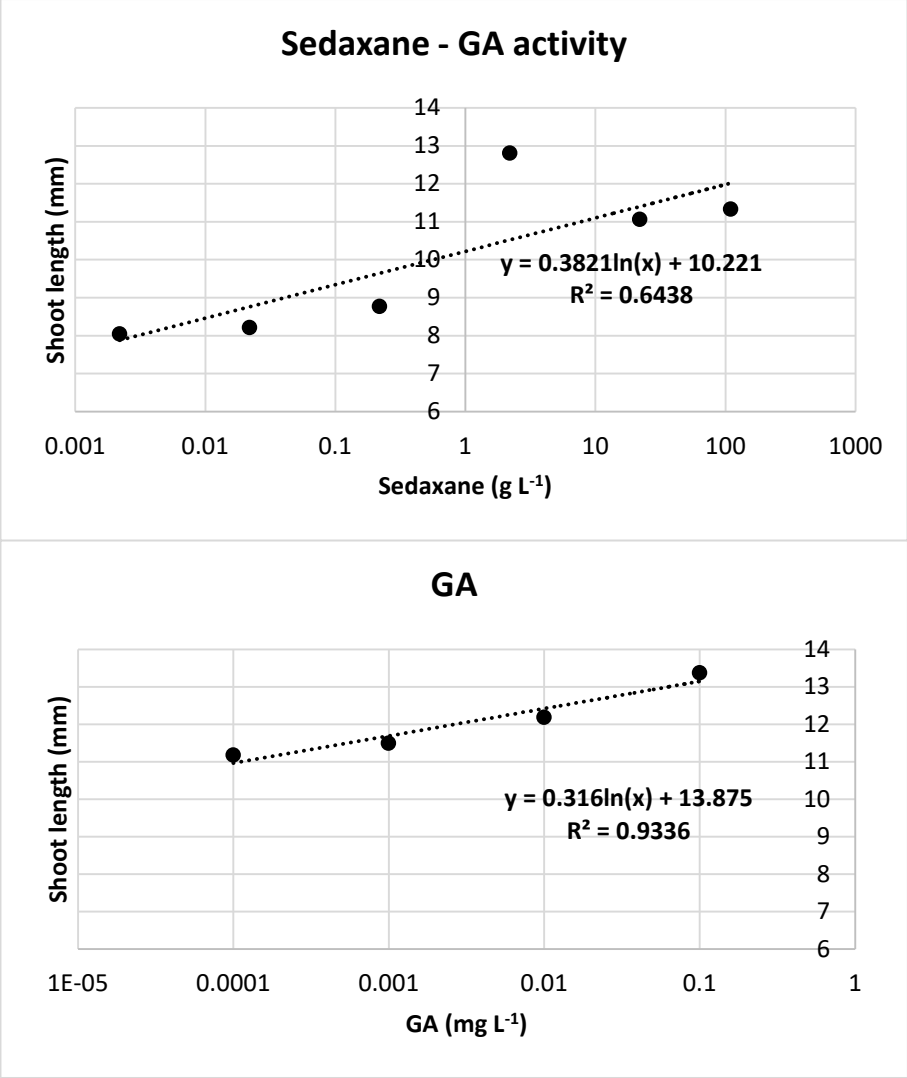
Under sterile conditions, fungicide treatment did not significantly enhance plant growth, although the medium dose of sedaxane (75 µg seed^{-1}) appreciably increased shoot (+21%) and root (+10%) biomasses as compared to untreated controls (Table 1). The effects of the seed treatments were more evident on other root features: Root length increased by 60% and root area by 45% at the intermediate fungicide dose. While root diameter was slightly smaller ($P > 0.05$), the number of root tips and forks increased, most noticeably with the intermediate (tips +27%, forks +51%) and maximum doses (tips +17%, forks +48%), although only the root branching increase was significant. These results show that root stimulation by sedaxane may be dose-dependent up to saturation.





	R^2	b	*P*
IAA	0.99	-1.76	0.00
Sedaxane	0.79	-0.197	0.02

Fig. 1 Audus test: auxin-like activity of sedaxane measured as root length variations in watercress. Linear regression analysis (below) performed on 20 samples and averaged over 5 replicates. Note that the x axis has a logarithmic scale.



	R^2	b	*P*
GA	0.93	0.72	0.04
Sedaxane	0.644	0.382	0.05

Fig. 2 Audus test: gibberellin-like activity of sedaxane measured as variation in shoot length in lettuce. Linear regression analysis (below) was performed on 20 samples and averaged over 5 replicates. Note that the x axis has a logarithmic scale.

Effects of sedaxane on SPAD, protein and sugar contents

Leaf greenness, measured in terms of SPAD values, was very stable across treatments at the end of the trial (Table 2), while protein content was significantly influenced by sedaxane ($P < 0.001$), with an increase of 14% at the intermediate and highest AI doses (Table 2). A similar effect was found in the roots, with protein content increasing significantly at the highest AI dose (+20% *vs*. untreated controls).

Fungicide treatment did not affect the shoot and root glucose content, the former having an average concentration of 3374 μg g^{-1} FW, the latter 3766 μg g^{-1} FW. The only variation found with regard to fructose was that it was significantly reduced in the shoot at the lowest and highest sedaxane doses (-21% and -15%, respectively, *vs*. untreated controls) (Table 2).

Table 1 Main shoot and root parameters (mean ± SE; n = 3) in *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Sedaxane dose (µg seed^{-1})	Shoot	Root					
	DW (g plant^{-1})	DW (g plant^{-1})	Length (m plant^{-1})	Area (m^2 plant^{-1})	Diameter (mm)	Tips (n plant^{-1})	Forks (n plant^{-1})
0	0.50±0.07^{a}	0.24±0.03^{a}	130.2±35^{b}	0.23±0.04^{b}	1.81±0.13^{a}	6657±1769^{a}	10594±2280^{a}
25	0.52±0.04^{a} (+3)	0.25±0.03^{a} (+3)	167.4±6^{ab} (+29)	0.26±0.01^{ab} (+14)	1.54±0.03^{a} (–15)	6180±957^{a} (–7)	12649±1681^{ab} (+19)
75	0.61±0.04^{a} (+21)	0.26±0.01^{a} (+10)	208.0±24^{a} (+60)	0.33±0.03^{a} (+45)	1.59±0.10^{a} (–12)	7784±994^{a} (+17)	15985±1849^{b} (+51)
150	0.53±0.04^{a} (+6)	0.24±0.01^{a} (+1)	184.9±16^{ab} (+42)	0.31±0.02^{ab} (+37)	1.68±0.10^{a} (–7)	8467±405^{a} (+27)	15711±718^{b} (+48)

Table 2 Leaf SPAD values, shoot and root protein, glucose, and fructose contents (mean ± SE; n = 9) in *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Sedaxane dose (µg seed^{-1})	Shoot				Root		
	SPAD	Protein (mg g^{-1} FW)	Glucose (µg g^{-1} FW)	Fructose (µg g^{-1} FW)	Protein (mg g^{-1} FW)	Glucose (µg g^{-1} FW)	Fructose (µg g^{-1} FW)
0	34.6±0.8^{a}	5.6±0.1^{b}	3328±170^{a}	1005±24^{a}	1.5±0^{b}	3819±128^{a}	1322±56^{a}
25	34.1±0.2^{a} (-1)	5.9±0.1^{ab} (+5)	3310±33^{a} (–1)	792±35^{b} (–21)	1.5±0.1^{b}	3724±163^{a} (–2)	1418±109^{a} (+7)
75	34.7±0.9^{a} (+0.5)	6.4±0.2^{a} (+14)	3340±113^{a}	1012±59^{a} (+1)	1.5±0.1^{b}	3665±140^{a} (–4)	1476±125^{a} (+12)
150	34.3±0.8^{a} (–0.5)	6.4±0.2^{a} (+14)	3518±70^{a} (+6)	855±14^{b} (–15)	1.8±0^{a} (+20)	3859±384^{a} (+1)	1313±191^{a} (–1)

Variations of GS and GOGAT activities with sedaxane

Glutamine synthetase (GS) activity and glutamate synthase (GOGAT) activity were, respectively, 3.8 and 2.1 times higher, on average, in the shoots than in the roots. Seed treatment with sedaxane significantly increased GS activity in the shoots ($P < 0.01$) at the lowest (+145% *vs*. controls) and intermediate AI doses (+45%), and in the roots ($P < 0.001$), particularly at the intermediate and highest AI doses (both +66%, $P \leq 0.05$) (Fig. 3).

Sedaxane treatments did not affect GOGAT activity in the shoots, while slight, but insignificant, reductions were observed in the roots (Fig. 3).

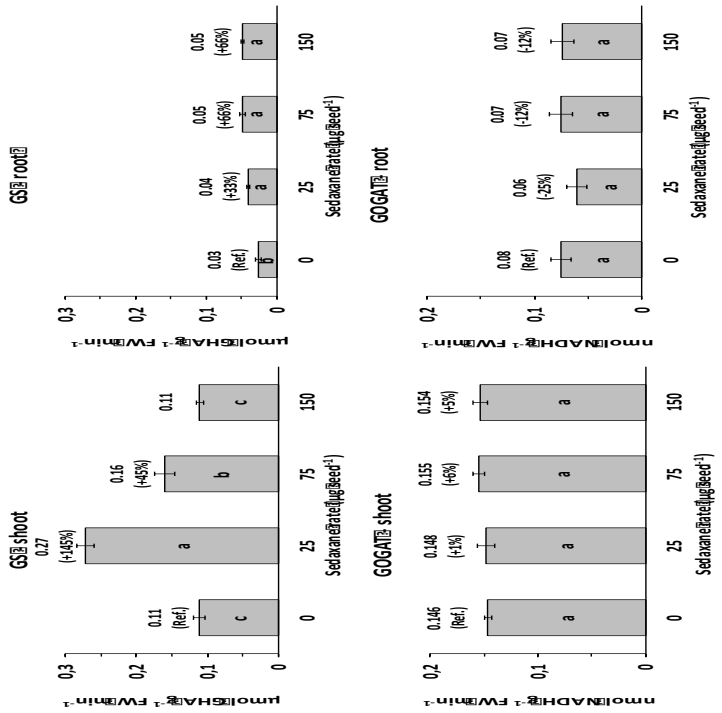


Fig. 3 Shoot and root glutamine synthetase (GS) and glutamate synthase (GOGAT) activities (mean ± SE; n = 9) in *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Effect of sedaxane on leaf phenylpropanoid metabolism

A significant increase in soluble phenolic acids in the shoots was observed at the lowest sedaxane dose (+14% *vs*. untreated controls), while values similar to controls were detected at greater AI doses ($P \leq 0.05$) (Table 3). However, when individual compounds were analyzed, large differences among treatments were detected for caffeic acid, and, to a lesser extent, for syringic and *p*-coumaric acids ($P \leq 0.05$). Significantly higher concentrations of caffeic acid were found in all treated plants compared with untreated controls ($P \leq 0.05$). Sedaxane increased caffeic acid by 41-58%, depending on the dose, and *p*-coumaric acid, the most abundant phenolic compound, by 23% at the lowest and 19% at the intermediate dose. There were only slight differences in the vanillic and *t*-ferulic acid contents in treated plants as compared with controls ($P > 0.05$).

The ANOVA revealed a significant increase ($P \leq 0.05$) in PAL enzyme activity in the shoots with the lowest and highest fungicide doses (+29% and +43%, respectively) as compared with untreated controls (Table 3).

Table 3 Shoot phenylalanine ammonia-lyase activity (PAL), soluble phenol content and phenolic acid profiles (mean ± SE; n = 9) of *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Sedaxane dose (µg seed^{-1})	PAL (nmol cinn. acid mg^{-1} prot. min^{-1})	Soluble phenols (as mg gallic acid g^{-1} FW)	Vanillic acid (µg g^{-1} FW)	Caffeic acid (µg g^{-1} FW)	Syringic acid (µg g^{-1} FW)	*p*-coumaric acid (µg g^{-1} FW)	*t*-ferulic acid (µg g^{-1} FW)
0	3.1±0.12^{b}	36.4±1.5^{b}	0.78±0.06^{a}	2.88±0.24^{b}	11.7±0.6^{b}	21±0.9^{b}	0.72±0.04
25	3.99±0.28^{a} (+29)	41.4±0.8^{a} (+14)	0.63±0.06^{a} (-19)	4.14±0.24^{a} (+44)	14.6±0.9^{a} (+25)	25.8±1.9^{a} (+23)	0.83±0.07 (+15)
75	3.08±0.22^{b} (-1)	35.1±1^{b} (-4)	0.63±0.03^{a} (-19)	4.55±0.43^{a} (+58)	13.8±0.6^{ab} (+18)	24.9±0.6^{a} (+19)	0.82±0.03 (+14)
150	4.42±0.26^{a} (+43)	35.2±2^{b} (-3)	0.63±0.05^{a} (-19)	4.06±0.34^{a} (+41)	12.4±0.6^{ab} (+6)	21.9±1^{b} (+4)	0.74±0.03 (+3)

Discussion

Sedaxane belongs to the new class of succinate dehydrogenase inhibitors, and is currently used as a seed-coating fungicide on various crops in several countries, with registration approval being increasingly granted worldwide. It is a broad-spectrum antifungal agent, and is of particular interest in combatting *Rhizoctonia solani* and *Mycosphaerella reliana* in maize.

In light of previous results on root stimulation in wheat (Barchietto et al. 2012), we investigated the side-effects of sedaxane in maize over and above its protective capacity, and found that seed treatment significantly modified morphological traits and physiological activities in disease-free plants grown in sterile soil.

The Audus test is considered to be the most reliable bioassay in terms of reproducibility and repeatability for verifying and quantifying the biostimulant activity of molecules in plants, and can be used to ascertain whether an exogenous compound has auxin- and/or gibberellin-like activity (Conselvan et al. 2017). Auxin (IAA) is the most important hormone in plants, and is involved in several plant growth and development phases, such as embryogenesis, organogenesis, tissue patterning and tropisms (Davies 2010). Molecular genetic studies have brought to light the central role of auxin in primary root elongation, lateral root initiation, and root hair development (De Smet et al. 2006; Overvoorde et al. 2010). The phytohormone gibberellin (GA) also modulates plant development by lengthening roots and stems, and expanding leaves (Fleet and Sun 2005). We used an Audus bioassay to demonstrate that sedaxane has both auxin- and gibberellin-like activity, as the confirmation of its biostimulant properties.

Although the improvements in aerial and root biomasses detected in this trial were not significant, we found that root length and area, and the number of root tips and branches increased almost in proportion to the dose of sedaxane, consistent with results reported by Colla et al. (2014) on maize coleoptile elongation with protein hydrolysates. All these root morphology modifications are known responses to biostimulant compounds (Calvo et al. 2014). Root development is essential for plant survival as it plays a crucial role in water and nutrient acquisition for growth, the synthesis and accumulation of secondary metabolites, and interaction with soil organisms (Saini et al. 2013).

The data collected from this trial are consistent with Barchietto et al. (2012) regarding stimulation of wheat shoots and roots by seed-applied sedaxane. At 30 days after sowing

(DAS), they observed significant increases in root length in treated plants as compared with controls, and no differences in root biomass, as in our case study at 20 DAS. Interestingly, they also found that at 60 DAS root length was unaffected by sedaxane seed treatment, whereas root biomass increased significantly (+39-87%, according to variety).

In the sterile soil conditions of our trial, the SPAD value was very stable across treatments, but this was not the case in the supplementary trial we carried out in unsterile soil conditions to investigate the potential effect of sedaxane in field-like conditions, where we found a slight but significant increase in SPAD (up to 7%) (Table SM1). This result is in line with practical expectations in the field given the correlation between SPAD and photosynthetic activity, the N status of the plant and protein contents (Prost and Jeuffroy 2007; Sim et al. 2015).

It should be noted that sedaxane may affect not only fungal mitochondria but also the SDH complex II of plants, partially inhibiting its activity (Avenot and Michailides 2010). Fuentes et al. (2011) reported better photosynthetic performance in *Arabidopsis* plants with compromised expression of the flavoprotein subunit of SDH than in wild-type plants. Inhibition of the SDH subunit also resulted in an increase in the number and aperture of leaf stomata, which significantly increased CO_2 assimilation, in turn enhancing growth and protein production. Araújo et al. (2011) obtained similar results with tomato plants with antisense inhibition of the iron-sulfur subunit of SDH. However, the higher SPAD values of sedaxane-treated maize observed in our supplementary study with unsterilized soil may also be related to a slowing down of chlorophyll molecule degradation, as reported for fungicides of the Strobilurin class (Grossmann and Retzlaff 1997; Xu and Huang 2009). However, this hypothesis needs to be confirmed by studying SDH activity and chlorophyll content in sedaxane-treated plants.

The higher protein content in sedaxane-treated seedlings may be ascribed to better nitrogen metabolism through the activity of the enzymes involved. In fact, the GS/GOGAT metabolic pathway is the main route of N assimilation in higher plants (Mokhele et al. 2012), allowing ammonium taken up directly or originating from nitrate to be assimilated into amino acids (Xu et al. 2012). The GS enzyme is also critical for re-assimilation of the NH_4^+ constantly released in large amounts via photorespiration, phenylalanine consumption for lignin biosynthesis, and protein catabolism (Lea and Miflin 2011). GS activity, which increased significantly following sedaxane application, therefore plays a pivotal role in many aspects of plant development (Seabra and Carvalho 2014), as it is a key component in nitrogen use efficiency (NUE) and plant yield (Thomsen et al. 2014).

GS and GOGAT enzyme activities have been previously reported to be affected by biostimulants (Baglieri et al. 2014). Our data are consistent with those of Ajigboye et al. (2016), who found that improvements in the photosynthetic efficiency, growth, and biomass of sedaxane-treated wheat plants were associated with up- or down-regulation changes in gene expression, and consequent modifications of physiological processes, particularly under drought stress conditions. In particular, sedaxane is reported to induce transcriptional regulation of genes and transcriptional factors, altering the flavonoid and phenolic metabolism (Ajigboye et al. 2016). Our study confirmed that sedaxane stimulates phenylpropanoid metabolism in maize as we found an increase in PAL enzyme activity, although, unexpectedly, the effect was not observed at the intermediate dose. The PAL enzyme catalyzes the first metabolic step from primary to secondary metabolism (Douglas 1996), deaminating phenylalanine to produce cinnamic acid. As a consequence, there was an increase in the total content of phenolic compounds in shoot tissues from seedlings treated with the lowest concentration of sedaxane, but not at the highest dose. However, there were substantial changes in the concentrations of individual phenolic acids in relation to fungicide application: In particular, there was a considerable increase in caffeic acid in treated plants, which may be of interest in view of its weak auxin-like effect (Ishikura et al. 2001; Lavee et al. 1986; Nagasawa et al. 2016). The main precursor of lignin in the cell wall of gramineous plants is *p*-coumaric acid, and a greater abundance of it in sedaxane-treated plants could contribute to more intense cell activity and division. Vanillic and *p*-coumaric acids are also reported to be antifungal phenols, meaning that sedaxane may also contribute indirectly to plant defense (Lattanzio et al. 2006; Pusztahelyi et al. 2015; Zabka and Pavela 2013). Stimulation of the secondary metabolism may also be explained by enhanced primary metabolism activity, as evidenced by the protein and sugar contents (Table 2).

As with other SDHIs studied in wheat, all the physiological changes brought about by sedaxane may also delay senescence, and improve the yield and protein content of maize plants (Abdelrahman et al. 2017; Bayles 1999; Dimmock and Gooding 2002; Zhang et al. 2010), but this requires further investigation in current field conditions.

We conclude that sedaxane has a considerable effect on rooting power of maize, particularly on the length, surface area and number of lateral roots. This study found that sedaxane exhibits biostimulant activity in maize seedlings due to its hormone-like activities, corroborated by the fact that most of the observed effects are saturated at moderate doses, as with phytohormones.

We have high expectations that seed treatment with this fungicide will facilitate plant establishment, and may provide particular benefits under adverse soil and climatic conditions. Stimulation of the enzyme activities involved in N assimilation and phenylpropanoid metabolism is in agreement with previous findings on this active ingredient and other SDHI fungicides, and is consistent with improved N status and antioxidant activity.

As the fungicide doses tested here are within the recommended label range, the biostimulant activity of sedaxane is an additional benefit, over and above its protective role against seed- and soil-borne diseases, which could be exploited in the cultivation of maize. Although further studies are needed to see whether these improvements also influence final growth and yield, our preliminary results suggest that, as things currently stand, roots may be enhanced in the early growth stages, even in non-sterile soil.

Contributions

CDC oversaw the greenhouse trial, assisted with the laboratory analyses, collected and analyzed the data, and wrote the first draft of the manuscript; GBC performed all the enzymatic and biological assays, and also collected and analyzed the data, carried out the literature research and improved the manuscript content; GB performed the HPLC analysis and assisted with analysis of the statistical data; PC helped design the experiment, analyzed the data and improved the manuscript content; LS helped revise the text; TV conceived the research idea, and corrected and arranged the final version of this work. All authors contributed to the interpretation and discussion of the results.

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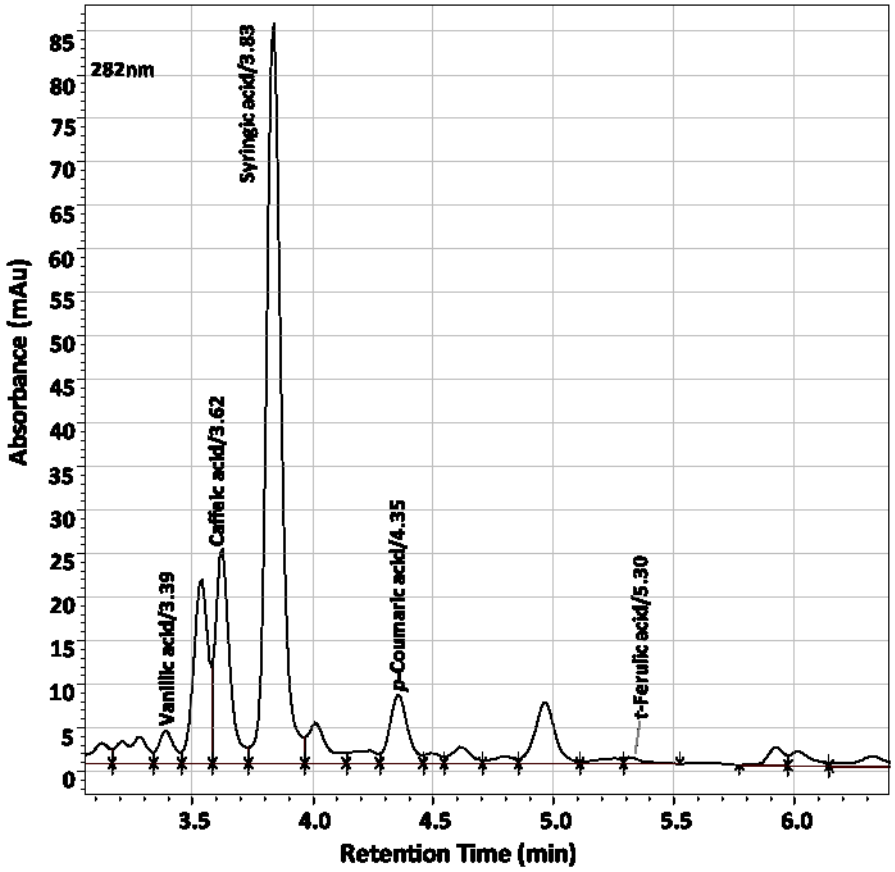
Supplementary material

Table SM1 Main shoot and root parameters (mean ± SE; n = 3) in *Zea mays* at 20 days after sowing (DAS) in unsterilized pot soil at increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs.* untreated controls.

Sedaxane dose (µg seed^{-1})	Shoot		Root					
	DW (g plant^{-1})	SPAD	DW (g plant^{-1})	Length (m plant^{-1})	Area (m^2 plant^{-1})	Diameter (mm)	Tips (n plant^{-1})	Forks (n plant^{-1})
0	0.35±0.03^{a}	27.9±0.7^{b}	0.17±0.01^{a}	137.4±9^{b}	0.21±0.01^{b}	1.55±0.05^{a}	4285±215^{b}	9798±1010^{a}
25	0.34±0.05^{a} (-3)	28.4±0.6^{ab} (+2)	0.17±0.01^{a}	155.5±21^{ab} (+13)	0.23±0.03^{ab} (+10)	1.49±0.01^{ab} (-4)	3804±618^{b} (-11)	10111±1277^{a} (+3)
75	0.42±0.02^{a} (+20)	28.4±0.7^{ab} (+2)	0.19±0.005^{a} (+12)	156.6±11^{ab} (+14)	0.23±0.02^{ab} (+10)	1.50±0.04^{ab} (-3)	4190±399^{b} (-2)	10298±796^{a} (+5)
150	0.42±0.04^{a} (+20)	29.9±0.6^{a} (+7)	0.20±0.01^{a} (+18)	189.1±19^{a} (+38)	0.27±0.02^{a} (+29)	1.43±0.03^{b} (-8)	5479±131^{a} (+28)	11628±958^{a} (+19)



Supplementary Figure 1 Sample chromatogram of phenolic aid HPLC determination. The mobile phase was 0.25% (v/v) trifluoroacetic acid (TFA, solvent A) and pure ACN (solvent B). The HPLC gradient was linear: after 2 μL sample injection, solvent B was kept at 4% for 1.16 min, then increased gradually to 12% in 1.16 min, to 23% in 4.63 min, to 95% in 1.85 min, to 100% in 1.16 min, and the final rate was maintained for a further 2.78 min. Analysis had a duration of 11.58 min at a solvent flow rate of 1.1 mL min-1. The HPLC equipment (Shimadzu, Kyoto, Japan) had a UV diode array detector (SPD-M20A) at wavelength 282 nm, and an Ultra Tech sphere C18 analytical column (33 mm × 4.6 mm i.d., 1.5 μm particle size; Cil Cluzeau, Sainte-Foy-La-Grande, France) kept at 36 °

7 General Conclusions

This Ph.D. project evaluated the biostimulant activity of humic substances (HS) and of a fungicide on the metabolism of agro-food plants.

It is well documented that HS increase plant growth, root system, and several physiological processes. These responses are a result of a complex network of mechanisms of action and there is an urgent need to better elucidate the causal/functional mechanism of HS.

In the first case of study, the proteomic approach revealed its potential to disclose the complexity of the plant metabolic response to HS. In treated roots of Arabidopsis plants, it was possible to observe that HS from heartworm *faeces* influenced the expression of enzymes involved in the energy metabolism and respiration, which have essential functions for various cellular processes such as biosynthesis of macromolecules (proteins, nucleic acids, amino acids, fatty acids, secondary metabolites). An acceleration of energy processes resulted in a higher ROS production and consequent up-regulation of ROS scavenging enzymes. Higher growth rate of roots was also evidenced by increased the expression of proteins involved in cell trafficking, cell division and protein production.

These results were confirmed by the metabolomics study, where we could analyse and compare sugars and amino acids profiles of Arabidopsis plants treated with HS extracted from different sources. We observed that different humic compounds induced different responses on test plants and the responses are only partially justified by the presence of hormones in the HS matrix. We could observe that plants under HS treatment had a significant reduction of carbohydrates and free amino acids concentrations in roots to support the stimulation of protein and energetic metabolism, which uphold the higher growth rate.

Similar results were also observed in the third case of study, where the biostimulant activity of HS extracted from four leonardites was tested. On treated maize seedlings, HS positively stimulated the elongation and proliferation of secondary roots, and the production of total soluble proteins. These effects were also supported by an enhancement of nitrogen metabolism, with an increment of GOGAT and GS enzymes activity. It was also observed a higher accumulation of phenolic compounds and higher activity of PAL enzyme. As in the proteomic study, the stimulation of this secondary metabolism pathway could be a response to the higher energy production and growth rate.

These three studies contributed to improve the knowledge on the mechanisms of action of HS. Even if HS were extracted from different sources were applied on different plant species, they displayed similar biological activities. Proteomics and metabolomics studies confirmed that "–omics" techniques are essential tools to have a 'panoramic' view on metabolic changes happening inside an organism after a positive or negative external perturbation.

In the last chapter of the Ph.D. project, we investigated the potential biostimulant activity of sedaxane, a succinate dehydrogenase inhibitor fungicide, on maize seedling. Under sterile conditions, sedaxane enhanced length and growth of roots, with a contemporaneous stimulation of proteins production, nitrogen and phenols metabolism. These additional benefits of the studied fungicide, could facilitate plant establishment, especially when soil and climate conditions are adverse. This suggests that biostimulant activity might not be relegated to humic or humic-like compounds, but different substances might induce positive responses in plants.

Future research will be oriented in better elucidating the relationship between biostimulants chemical structure and plant physiological responses, also by means of proteomics and metabolomics analyses. Further studies should address the biostimulant responses in field and natural environments and in plants under stress condition.

This could help for developing a new generation of biostimulants from natural resources or from other sources such as actual pesticides.